From DEPARTMENT OF MOLECULAR MEDICINE & SURGERY
Karolinska Institutet, Stockholm, Sweden

APPETITE CONTROL - GENETIC, IMMUNOLOGICAL AND NEUROBIOLOGICAL ASPECTS

Charlotte Lindfors

Karolinska Institutet

Stockholm 2017

About the cover page: The image is drawn by 4-year-old Linnea Lindfors, and according to her own description the image consists of one small sad mouse and one big happy mouse. To me the “small sad mouse” is the *anx/anx* mouse, and “the big happy mouse” is the wild-type.

Published by Karolinska Institutet.
Printed by Eprint AB 2017

ISBN 978-91-7676-631-6

APPETITE CONTROL - GENETIC, IMMUNOLOGICAL AND NEUROBIOLOGICAL ASPECTS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

For the degree of PhD at Karolinska Institutet

The thesis will be defended in public on Friday the 21st of April at 9 am in the CMM lecture hall, L8:00, Karolinska Hospital, Stockholm, Sweden

By

Charlotte Lindfors

Principal Supervisor:
Assistant Professor Ida Nilsson
Karolinska Institutet
Department of Molecular Medicine and Surgery

Co-supervisors:
Professor Martin Schalling
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Tomas Hökfelt
Karolinska Institutet
Department of Neuroscience

Professor Roger Adan
Department of Rudolf Magnus Institute of Neuroscience

Opponent:
PhD Marc Claret
Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)
Centre Esther Koplowitz (CEK)

Examination Board:
Professor Angelica Lindén Hirschberg
Karolinska Institutet
Department of Woman's and Children's Health

Associate Professor Maria Swanberg
Lunds University
Department of Experimental Medical Science

Associate Professor Jorge Ruas
Karolinska Institutet
Department of Physiology and Pharmacology

ABSTRACT

The overall goal of this thesis is to develop a better understanding of the brain's regulation of appetite, i.e. the hypothalamic regulation of hunger and satiety signals. The correct regulation of appetite is crucial, potentially involved both in traditional eating disorders, such as anorexia nervosa and bulimia nervosa, as well as overweight, obesity and failure to thrive in infants and children. Anorexia/cachexia is also a severe and frequent complication to several common disorders such as cancer, HIV, renal failure and Alzheimer's disease and is one of the most important factors determining both quality of life and mortality in these conditions.

In this thesis, the *anx/anx* mouse is used as a tool to develop a better understanding of the hypothalamic regulation of food intake in an anorectic condition. Previous studies on the *anx/anx* mouse hypothalamus showed several abnormalities in the expression of neurotransmitters and neuropeptides in the orexigenic NPY/AgRP and the anorexigenic POMC/CART system. In order to elucidate when this abnormal phenotype first appears, we studied the development of the NPY/AgRP system in **Paper I**. The development of the NPY/AgRP system in the *anx/anx* mice is normal until P12. From this day, a gradual decrease in fiber density is seen with the weakest expression at P21, results that clearly show an impaired development of the NPY/AgRP system in the *anx/anx* mouse. A second aim for **Paper I** was to study the underlying mechanisms for this impaired development. We therefore analyzed the expression of markers of activated microglia and detected a gradual increase in these markers in the same areas and at the same time points as was seen for the gradual decrease in density of AgRP fibers. The impaired development of the AgRP system and the overlapping activation of microglia in the hypothalamus of the *anx/anx* mouse indicate an inflammatory/degenerative process. This was further investigated in **Paper II**, where we detected expression of MHC class I mRNA and protein in the arcuate nucleus, both in glial cells and in neurons. We also found that the neurons expressing the MHC class I subunit are 'silenced' or have a very low activity. Further evidence for neurodegeneration was seen in **Paper II,** as we detected a significantly increased number of apoptotic cells in several hypothalamic areas of the *anx/anx* mouse, as well as a double-labeling of NPY and active caspase 6, a marker for axonal degeneration, in neuronal fibers. Altogether, these results point to a neurodegenerative process in the hypothalamus of the *anx/anx* mouse. In **Paper III**, we continued the search for the *anx* gene/mutation to find the causes leading to the anorectic phenotype. Through a microarray study and the following pathway analysis the first indications were given that the anorectic phenotype in the *anx/anx* mouse is related to mitochondrial dysfunction and oxidative stress. We also detected that the *anx* mutation leads to decreased expression of the *Ndufaf1* gene and

protein. This gene, located within the *anx* interval, encodes an assembly factor for mitochondrial complex I. We show that the downregulation of *Ndufaf1* is associated with the *anx*-allele and not due to a secondary effect of the starvation. We could also see that the downregulation of the *Ndufaf1* gene leads to a less completely assembled mitochondrial complex I and the accumulation of sub-complexes, as well as increased levels of reactive oxygen species, in the *anx/anx* hypothalamus. Based on these results, we concluded that the anorexia and premature death of the *anx/anx* mouse is related to mitochondrial dysfunction and oxidative stress. In **Paper V**, we evaluated whether the activity in the hypothalamus is attenuated in the *anx/anx* mouse as a consequence of the defect mitochondrial complex I. We could see that hypothalamic glucose uptake in the fasted state was reduced in the *anx/anx* mouse. Further, the *anx/anx* hypothalamus had elevated levels of one of the glucose transporters, GLUT4 and a key metabolic molecule, AMPK. However, the hypothalamic activation state of AMPK was significantly decreased. Finally, during metabolic stress, levels of both AMP and IMP (both breakdown products of ATP) were decreased, while ATP levels were increased in the *anx/anx* hypothalamus. Together these results indicate that the *anx/anx* mouse has a reduced hypothalamic metabolism. This may contribute to the anorectic behavior of this mouse i.e. its inability to regulate food intake in response to the energy status. In **Paper IV**, we evaluated whether the inflammation and the mitochondrial dysfunction in the hypothalamus of the *anx/anx* mouse also could be detected in the endocrine pancreas, and if glucose homeostasis is disturbed. We found a strong downregulation of the *Ndufaf1* gene, paralleled by a reduced mitochondrial complex I activity in isolated *anx/anx* islets. In addition, there was an increased macrophage infiltration in *anx/anx* islets, indicating inflammation. Moreover, elevated levels of free fatty acids were seen in *anx/anx* serum. In contrast, isolated islets from *anx/anx* mice cultured in the absence of free fatty acids did not show any inflammation. Also, an intraperitoneal injection of glucose to the *anx/anx* mouse revealed a marked glucose intolerance associated with reduced insulin release. However, the insulin release from isolated *anx/anx* islets was increased after stimulation either with glucose or KCl. The conclusion of **Paper IV** is that the *anx/anx* endocrine pancreas display marked reduction in insulin release that correlates with the increased serum levels of free fatty acids, and that the accompanying in vivo inflammation may lead to inhibition of insulin secretion.

LIST OF SCIENTIFIC PAPERS

I. Nilsson I, **Lindfors C**, Fetissov S, Hökfelt T, Johansen JE. Aberrant Agouti-Related Protein System in the Hypothalamus of the *anx/anx* Mouse Is Associated With Activation of Microglia. The Journal of Comparative Neurology, 2008, 507:1128-1140

II. Nilsson I, Thams S, **Lindfors C**, Bergstrand A, Cullheim S, Hökfelt T, Johansen JE. Evidence of Hypothalamic Degeneration in the Anorectic *anx/anx* Mouse. Glia, 2011, 59:45-57

III. **Lindfors C***, Nilsson I*, Garcia-Roves PM, Zuberi AR, Karimi M, Donahue LR, Roopenian DC, Mulder J, Uhlén M, Ekström T, Davisson MT, Hökfelt T, Schalling M, Johansen JE. Hypothalamic mitochondrial dysfunction associated with anorexia in the *anx/anx* mouse. PNAS, 2011, 108:18108-18113

IV. **Lindfors C**, Katz A, Selander L, Johansen JE, Marconi G, Schalling M, Hökfelt T, Berggren PO, Zaitsev S, Nilsson I. Glucose intolerance and pancreatic β-celldysfunction in the anorectic *anx/anx* mouse. Am J Physiol Endocrinol Metab, 2015, 309:E418-E427

V. Bergström U*, **Lindfors C***, Svedberg M, Johansen JE, Häggkvist J, Schalling M, Wibom R, Katz A, Nilsson I. Reduced metabolism in the hypothalamus of the anorectic *anx/anx* mouse. J Endocrinol. 2017, 233:15-24

* Equal contribution

ADDITIONAL PUBLICATIONS

I. Hökfelt T, Stanic D, Sanford SD, Gatlin JC, Nilsson I, Paratcha G, Ledda F, Fetissov S, **Lindfors C**, Herzog H, Johanssen JE, Ubink R, Pfenninger KH. NPY and its involvement in axon guidance, neurogenesis, and feeding. Nutrition, 2008, 24(9):860-868

II. Nilsson I, **Lindfors C**, Schalling M, Hökfelt T, Johansen JE. Anorexia and hypothalamic degeneration. Vitam. Horm. 2013, 92:27-60

CONTENTS

LIST OF ABBREVIATIONS

actCasp6	active caspase 6
AD	Alzheimer's disease
ADP	adenosine diphosphate
AgRP	agouti related neuropeptide
AM	acetoxymethyl
α-MSH	α-melanocyte-stimulating hormones
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AN	anorexia nervosa
anx	the anorexia gene/mutation
APAF1	the apoptotic protease activating factor 1
Arc	arcuate nucleus
ATP	adenosine triphosphate
β2m	β2 microglobulin
BMI	body mass index
BN	bulimia nervosa
BN PAGE	blue native PAGE
BNST	bed nucleus of the stria terminalis
$[Ca^{2+}]_i$	free cytosolic Ca^{2+} concentration
CART	cocaine-amphetamine-regulated transcript
CI-V	Complexes I-V
Cr	creatine
CS	citrate synthase
cyt c	cytochrome c
DA	dopamine
DAPI	4,6-diamidino-2phenylindole
DHE	dihydroethidium
DMH	dorsomedial hypothalamus
ELISA	enzyme-linked immunosorbent assay
$FADH_2$	flavin adenine dinucleotide
FCCP	carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FFAs	free fatty acids
FTT	failure to thrive

Ghsr	growth hormone secretagogue receptor
GHS-R1	growth hormone secretagogue receptor type 1
G6P	glucose 6-P
GPX	glutathione peroxidases
GLUT2	type 2 glucose transporter
GLUT4	type 4 glucose transporter
GWAS	genome wide association study
[^{3}H]	tritium
H_2O_2	hydrogen peroxide
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
Iba1	ionized calcium binding adapter 1
IHC	immunohistochemistry
IL-1β	interleukin 1β
IMP	inosine monophosphate
ip	Intraperitoneal
IPA	ingenuity pathway analysis
ipGTT	intraperitoneal glucose tolerance test
ir	immunoreactivity
ISH	in situ hybridization
$K^+_{\text{-ATP}}$	ATP-sensitive potassium channels
KRBB	Krebs-ringer bicarbonate buffer
Lepr	leptin receptor
LHA	lateral hypothalamic area
LS	Leigh syndrome
MC3-R	melanocortin receptor 3
MC4-R	melanocortin receptor 4
MHC	major histocompatibility complex
MMP	mitochondrial membrane potential
N_2	nitrogen
NADH	nicotinamide adenine dinucleotide
NBT	nitro blue tetrazolium
NDUFAF1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 1
NO	nitric oxide

NTRK3	neurotrophin receptor tyrosine kinase 3
O_2^-	superoxide
OXPHOS	oxidative phosphorylation system
PCr	phosphocreatine
PD	Parkinson's disease
POMC	pro-opiomelanocortin
PRDX	peroxiredoxins
PVN	paraventricular nucleus
Q	ubiquinone
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gels
SOD	superoxide dismutase
SSLP	sequence-length polymorphism
T1D	type 1 diabetes
T2D	type 2 diabetes
TLR2	toll-like receptor 2
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
TSA	tyramide signal amplification
TUNEL	Terminal dUTP Nick End Labeling
Tyro3	tyrosine receptor kinase 3
VMH	ventromedial hypothalamus
Wt	wild-type
Y1r	neuropeptide Y1 receptor

1 BACKGROUND

1.1 EATING DISORDERS

A functional energy homeostasis, that is an accurate regulation of the balance between energy intake and energy expenditure, is essential from several points of view. In fact, a dysfunctional regulation of energy homeostasis is a severe complication associated with many common diseases. One way to disrupt energy balance is a disturbed appetite, resulting in excessive or severely reduced food intake. This can lead to overweight and obesity or eating disorders, such as anorexia nervosa (AN) or bulimia nervosa (BN). In turn, overweight and obesity increase the risk for type 2 diabetes (T2D), cardiovascular disorders [1], and certain types of cancers, e.g. colon cancer [2]. On the other hand, anorexia, meaning poor appetite, is not just a term referred to the severe eating disorder AN, but is also a frequent complication of many somatic diseases such as cancer, HIV, renal failure, inflammatory disorders, chronic kidney disease, chronic heart failure, and Alzheimer's disease [3-5]. This severe state of malnutrition, called cachexia, is a condition of nutrient imbalance in combination with an already severe disease, and is a consequence of a decreased appetite combined with an increased energy expenditure [6]. In this situation, when the body is already struggling against a disease, the severity of cachexia is one important factor determining both quality of life and mortality. In fact, in AIDS patients retaining body mass retention has the strongest association with survival [7, 8].

Another anorectic condition is called failure to thrive (FTT), also known as weight faltering. That is a term used to describe a young child or infant with insufficient weight gain in relation to its age and gender, and is caused by undernutrition in relation to this child's specific energy requirements [9, 10]. Non-organic FTT is when faltering growth is seen, and no organic causes are found. These children fail to grow, because they consume insufficient calories, most of the time due to a decreased appetite, resulting in decreased caloric intake. It appears as if the hypothalamus, seen as the appetite center of the brain, is not in tune with the calorie requirement of the child [11, 12].

Another class of diseases with a primary alteration in the mechanisms controlling appetite and body weight are the eating disorders already mentioned, the most well known ones being AN and BN. Dieting and weight control are strongly associated with the onset of eating disorders. In fact, body dissatisfaction is the best-known contributor to the development of eating disorders [13]. Several studies show an increase of body dissatisfaction mostly among young women and children [14-16]. Most of the persons dieting will regain their weight within a few

years; however, studies have shown that 35% of persons dieting will continue to a pathological dieting and of those 20-25% will develop an eating disorder [17].

Another group of individuals showing an increased risk of developing an eating disorder is individuals with type 1 diabetes (T1D) [18, 19]. Some individuals with diabetes intentionally reduce or omit insulin to control weight. This manipulation of insulin coupled with eating disorders behaviors is termed diabulimia [20]. The numbers vary, but studies show a range between 30-40% of individuals with T1D who also develop unhealthy weight controls, such as manipulating weight with insulin, or meet the criteria for an eating disorder [21]. This is an unhealthy combination that results in poor metabolic control. The individual not only struggle with the complications due to the eating disorder, but also eventually develops diabetes-related complications.

AN is a serious form of eating disorder characterized by self-starvation leading to an excessive weight loss. This is also combined with a strong fear of gaining weight as well as an inability to understand the severity of the situation (DSM-V). During the progress of AN, the body does not, for a longer period, receive sufficient nutrients, resulting in a weakened body and an inability of the body to function normally. There are severe consequences of this, since the body is forced to slow down all its necessary processes to save energy, resulting in e.g. slow heart rate, low blood pressure and muscle loss [22]. In addition, among individuals suffering from an eating disorder there is an increased risk for depression [23, 24], and AN has the highest death rates of all psychiatric disorders with a mortality rate as high as 10% [25-29].

As for today, the causes for AN are to a large extent not known. We do know that additive genetic factors account for 50-80% of the background [3, 28], but we still lack knowledge of which these genetic variants are. A genome wide association study (GWAS) recently reported the first genome-wide significant locus for AN at a site previously implicated in T1D (http://dx.doi.org/10.1101/088815). They also report significant negative SNP-based genetic correlations between AN and body mass index (BMI) and cardiometabolic parameters. Three genetic loci involved in both AN and BMI was also detected in another study [30]. These findings suggest that AN should be seen as a disorder not only with psychiatric but also metabolic components. However, due to lack of knowledge about, in particular, the molecular neurobiological pathways involved in the development and progress of AN, we face a situation where the best treatment methods still need to be found. The most important factor for full recovery from AN is early treatment. The probability of a full recovery from AN depends on the duration of the condition [25-29]. This underlines the need to further study and better

understand the mechanisms underlying appetite control and to find new methods for treatment to obtain full recovery from different kinds of eating disorders.

This thesis is based on the anorectic *anx/anx* mouse (to be described fully in a separate section below), a unique, genetic mouse model for studying appetite control and disturbed feeding behavior. The *anx/anx* mouse shows similar phenotypes with several conditions of dysregulated appetite such as AN [4], cachexia [8] and FTT [31]. Studying this model may thus give us new insights into severe eating conditions, including a dysregulated appetite, and may lead us to new and better methods for the treatment of this conditions.

1.2 HYPOTHALAMIC REGULATION OF FOOD INTAKE

Hypothalamus is the site for several neuronal networks regulating appetite and body weight, i.e. the control of initiation and termination of food intake. This is also the place for regulation of glucose and energy homeostasis [32-49]. These networks can directly sense metabolic information from the periphery and then convert this information to metabolic signals of hunger or satiety [50].

In the arcuate nucleus (Arc) of the hypothalamus, two groups of neurons are located with opposite effects on feeding, e.g. hunger and satiety signaling neurons [51]. The satiety signaling group of neurons express two anorexigenic peptides, pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) [52-55]. The POMC precursor is cleaved into α-melanocyte-stimulating hormone (α-MSH) which, upon stimulation, reduces food intake and increases energy expenditure [56, 57]. The hunger signaling group of neurons co-expresses two orexigenic neuropeptides, neuropeptide Y (NPY) [58-60] and agouti related neuropeptide (AgRP) [61-65] which stimulate food intake and reduce energy expenditure upon activation [66, 67].

Both the anorexigenic and the orexigenic groups of neurons are regulated by circulating metabolic hormones such as leptin, insulin, and ghrelin which bind to their corresponding receptors to transmit their specific response of hunger or satiety [68-70]. Insulin, produced by the β-cells, and leptin, secreted by adipocytes, circulate at levels proportional to body fat and enter the brain in proportion to their plasma levels. Both insulin and leptin act as anorexigenic hormones by stimulating POMC/CART neurons and inhibiting AgRP/NPY neurons, resulting in decreased food intake [71]. Ghrelin is a protein secreted from the stomach and is released during fasting. It activates the orexigenic AgRP/NPY neurons in the Arc, thereby stimulating food intake [72-74] (Fig. 1).

Both the POMC/CART and AgRP/NPY neurons project further to other hypothalamic areas, such as ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA) as well as brain regions outside of the hypothalamus, such as the bed nucleus of the stria terminalis (BNST) [39, 75-81].

Melanocortin receptor 4 (MC4-R) is found in neurons in the PVN [82] and decreases food intake and increases energy expenditure upon activation. MC4-R is activated by high levels of α-MSH, and by decreased levels of NPY, and of AgRP that works as an antagonist at the MC4-R [83, 84]. High levels of insulin and leptin activate POMC/CART and suppress NPY/AgRP, resulting in increased activity of MC4-R. Activated MC4-R results in suppressed activity of AMP-activated protein kinase (AMPK), a sensor of energy depletion that upon activation increases appetite, and thereby leads to decreased food intake and increased energy expenditure [84].

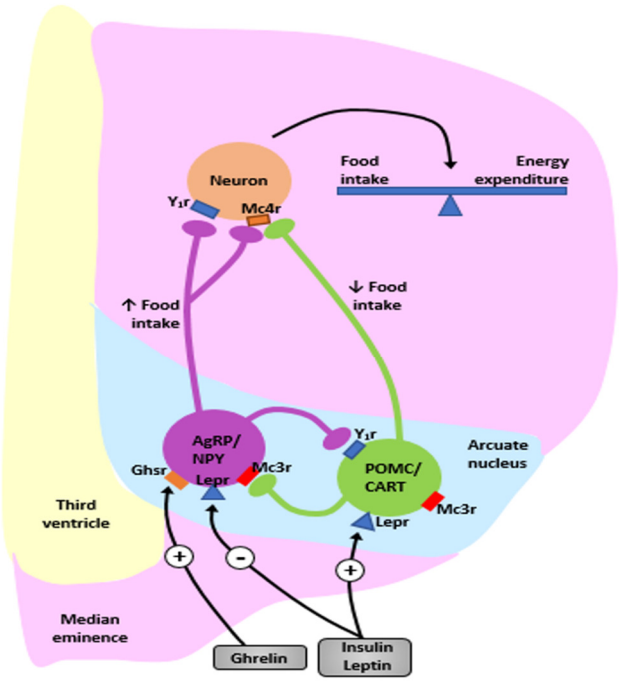

Fig. 1 Schematic overview of the regulation of food intake via peripheral metabolic signals, such as leptin, insulin and ghrelin, to the arcuate neurons NPY/AgRP and POMC/CART. Insulin and leptin inhibit AgRP/NPY neurons and stimulate POMC/CART neurons. Ghrelin can activate AgRP/NPY neurons and thereby stimulate food intake. AgRP/NPY neurons stimulate food intake and decrease energy expenditure and POMC/CART neurons inhibit food intake and increase energy expenditure. Ghsr, growth hormone secretagogue receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor; Y1r, neuropeptide Y1 receptor. Image modified from [85]

1.2.1 Hypothalamic AMPK – a regulator of energy homeostasis

AMPK is a sensor and regulator of energy homeostasis and protects the body against energy depletion by stimulating appetite when activated, i.e. AMPK is activated during starvation and inactivated during feeding [86, 87]. AMPK exerts its actions by stimulating the orexigenic neuropeptides and inhibiting the anorexigenic neuropeptides, leading to increased food intake. The activation of AMPK also results in reduced thermogenesis aiming at decreasing energy expenditure [84, 88].

The activation or inhibition of AMPK is regulated by several different factors e.g. the metabolic hormones insulin, leptin and ghrelin. Insulin and leptin are both inhibitors of AMPK, since both insulin and leptin levels increase after food intake [89, 90]. Insulin can reduce the activity of AMPK in all hypothalamic regions, whereas leptin inhibits AMPK activity in the Arc and PVN [84]. Ghrelin is an activator of AMPK, and increases appetite by the activation of AMPK in both VMH [91] and in Arc [92] via growth hormone secretagogue receptor type 1 (GHS-R1) [93, 94].

Another AMPK regulator is adenosine triphosphate (ATP). Low levels of ATP reflect a low total energy reserve. AMPK is activated in response to ATP depletion, which causes an increase in the adenosine monophosphate (AMP):ATP or adenosine diphosphate (ADP):ATP ratios [95-97]. Activation of AMPK promotes the production of ATP by stimulating catabolic [98, 99] and inhibiting anabolic processes requiring ATP [100-103].

AMPK can also sense the levels of glucose and thereby regulate glucose homeostasis. The activation of hypothalamic AMPK results in increased glucose production, and AMPK can thereby protect the brain against hypoglycemia. This is an important task for AMPK, since glucose is the main source of energy for the body and is important for normal brain activity. In fact, if blood glucose drops below normal levels, this will result in hypoglycemia, a condition that can lead to instability and abnormal function of the brain [104-108].

Finally, another stimulator of AMPK is through contraction of the skeletal muscles. Thus, intense exercise significantly increases AMPK activity in humans [109].

1.3 NEUROINFLAMMATION

Microglia are the immune cells of the brain with a protective role to seek and destroy pathogens and to clear the tissue from cellular debris. When microglia are in a resting state, they have a ramified appearance with long processes extended from the cell body. Microglia use these processes to ‘search’ the surrounding environment to identify pathogens [110, 111]. The resting

microglia have a low expression of major histocompatibility complex (MHC) proteins [112]. When detecting pathological changes, brain injury and damaged or dead cells, microglia become activated and change their morphology into an amoeboid shape [113]. The activated microglia migrate to the site of the injury, where they phagocytose and destroy pathogens and remove damaged or dead cells. As a part of this response activated microglia release pro-inflammatory molecules, e.g. cytokines and chemokines, which help to elevate and direct the immune response, as well as cytotoxic factors, e.g. tumor necrosis factor-α (TNF-α), nitric oxide (NO) and reactive oxygen species (ROS), with the aim to destroy the pathogens [114-116]. However, microglia can also become neurotoxic by being chronically activated. If that happens, they become a chronic source of cytokines and ROS that will result in progressive neuron damage. This state of chronic and neurotoxic microglia activation has been implicated in the pathology of several neurodegenerative diseases [117].

The activation of microglia also results in increased expression of MHC molecules, giving microglia an antigen-presenting capacity. MHC Class I and II are cell surface glycoproteins that bind and present antigens to T lymphocytes [118]. MHC proteins can be expressed by both neurons and glial cells. Neurons express MHC Class I protein during acute inflammation and following changes in electrical activity [119-122], and glial cells express MHC class I and/or II during pathological conditions, e.g. multiple sclerosis and brain injury [123, 124].

1.3.1 Hypothalamic inflammation in relation to food intake

Hypothalamic inflammation can induce dysfunction in energy homeostasis and promote both obesity and anorexia. Low-grade inflammation in the hypothalamus suppresses the signals from insulin and leptin, leading to insulin and leptin resistance as well as increased food intake and decreased energy expenditure that promotes obesity. Several studies show a connection between hypothalamic low-grade inflammation and obesity [125-127]. However, a high-grade inflammation in hypothalamus results in decreased food intake and increased energy expenditure. Hypothalamic high-grade inflammation induces anorexia, and can result in the cachexia occurring in, e.g. cancer. It is the same inflammatory mechanisms that can induce both obesity and anorexia depending on the level of inflammation; however, the exact threshold when the condition of obesity changes to a condition of anorexia is not known [128]. There are indications that microglia may play a direct role in the regulation of food intake [129]. Thus, microglia activation in the brain is involved in both anorexia and body weight loss, as well as in the development of obesity.

1.3.1.1 Hypothalamic inflammation and microglia activation in obesity and anorexia

The accumulation of activated microglia in the hypothalamus has been linked to the development of obesity. Hypothalamic microglia are thought to be sensors of saturated fat, since they are shown to be activated by increased levels of free fatty acids (FFAs). During these conditions, e.g. the activation of microglia in the hypothalamus due to high intake of saturated fat, the activated microglia could be responsible for changes in hypothalamic functions, such as a decreased hypothalamic sensitivity to leptin, resulting in increased food intake. Several studies using animal models, where obesity is induced by high-fat diet (HFD), show this connection. In these animal models HFD induces inflammation and triggers the activation and accumulation of microglia in neurons in the Arc [130-132]. It is thought that the inflammation and microglia activation could be the cause of the neuronal injury and apoptosis seen in hypothalamic nuclei, both in rodents fed with HFD and in neurons treated with fatty acids [133, 134]. The activation of microglia cells and the neuronal injury results in dysfunction of hypothalamic neurons regulating food intake and energy homeostasis, that in turn can promote obesity [135].

Hypothalamic inflammation and microglia activation has also been linked to anorectic conditions. One study shows that anorexia and body weight loss developed as a result of activated microglia in the Arc. In an animal model, hypothalamic inflammation was triggered through the activation of toll-like receptor 2 (TLR2). This resulted in activation and accumulation of microglia in the Arc, and increased activity of POMC neurons, leading to an anorectic behavior in these mice [136].

In addition, the anorexia-cachexia syndrome has been associated with several pro-inflammatory cytokines released during inflammation, such as tumor necrosis factor (TNF) and interleukin 1β (IL-1β). These cytokines can be produced by neurons or glial cells in the CNS and can alter the function of several appetite regulating neurons in the Arc and, in this way, control the energy regulation in this syndrome [137-141]. This is also seen in animal models, where the anorexia-cachexia syndrome develops through administration of cytokines [142-146], and the symptoms of this condition are inhibited with the help of anti-TNF antibodies [145, 147, 148].

Furthermore, several approaches with the aim to inhibit the overall inflammation result in decreased hypothalamic inflammation and increased food intake in animal models of anorexia. To take a few examples, hypothalamic inhibition of AMPK reduced the hypothalamic inflammation and increased food intake in cancer-induced anorexia [149]. Also, in tumor-bearing rats it was shown that the hypothalamic inflammation was suppressed after physical

exercise [150]. And lastly, it was seen that the expression of IL1-β was reduced after ghrelin treatment, and this resulted in increased food intake [151, 152].

1.4 THE MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION SYSTEM

The mitochondrial oxidative phosphorylation system (OXPHOS), located in the mitochondrial inner membrane, is responsible for production of energy, i.e. the ATP required for the cells. This system consists of five complexes (CI-CV) and two electron carriers, ubiquinone (Q) and cytochrome c (cyt c). The first four OXPHOS complexes (CI-CIV) together with the two electron carriers, build up the electron transport chain and transfer electrons from the substrates nicotinamide adenine dinucleotide (NADH) (at CI) and flavin adenine dinucleotide ($FADH_2$) (at CII) through the chain to the final electron acceptor, molecular oxygen at CIV. The energy generated by this electron transfer is used to build up a gradient by protons being transferred across the mitochondrial inner membrane at CI, CIII, and CIV. The proton translocation results in a mitochondrial membrane potential that is used by complex V to generate ATP from ADP and inorganic phosphate (Fig. 2) [153].

1.4.1 Mitochondrial ROS production in the OXPHOS system

Mitochondrial ROS, e.g. superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are generated during the mitochondrial OXPHOS process. During normal conditions, the final electron acceptor at CIV, i.e. the oxygen molecule, is reduced to produce water. However, leakage of electrons, mainly from CI and CIII, leads to incomplete reduction of oxygen, to form superoxide, the precursor of most other ROS. The cell has an antioxidant defense system that can convert the different forms of ROS to less reactive forms and to water. Superoxide dismutases (SOD), glutathione peroxidases (GPX), and peroxiredoxins (PRDX) are all compounds belonging to the cells antioxidant defense system. There are three different isoforms of SOD localized in different places in the cell: copper-zinc SOD (CuZn-SOD or SOD1) localized in the mitochondrial intermembrane space and the cytosol [154], manganese SOD (Mn-SOD or SOD2) localized in the mitochondrial matrix, and an extracellular CuZn-SOD (EC-SOD or SOD3). Genetic elimination of Mn-SOD, in contrast to the other isoforms, is embryonically lethal [155, 156]. Thus, after the production of superoxide, the cell uses its antioxidant defense system to convert superoxide to hydrogen peroxide by the different isoforms of SOD. In the mitochondrial matrix, hydrogen peroxide is further detoxified by being fully reduced to water by, GPX1, PRDX3 or PRDX5 (Fig. 2) [157].

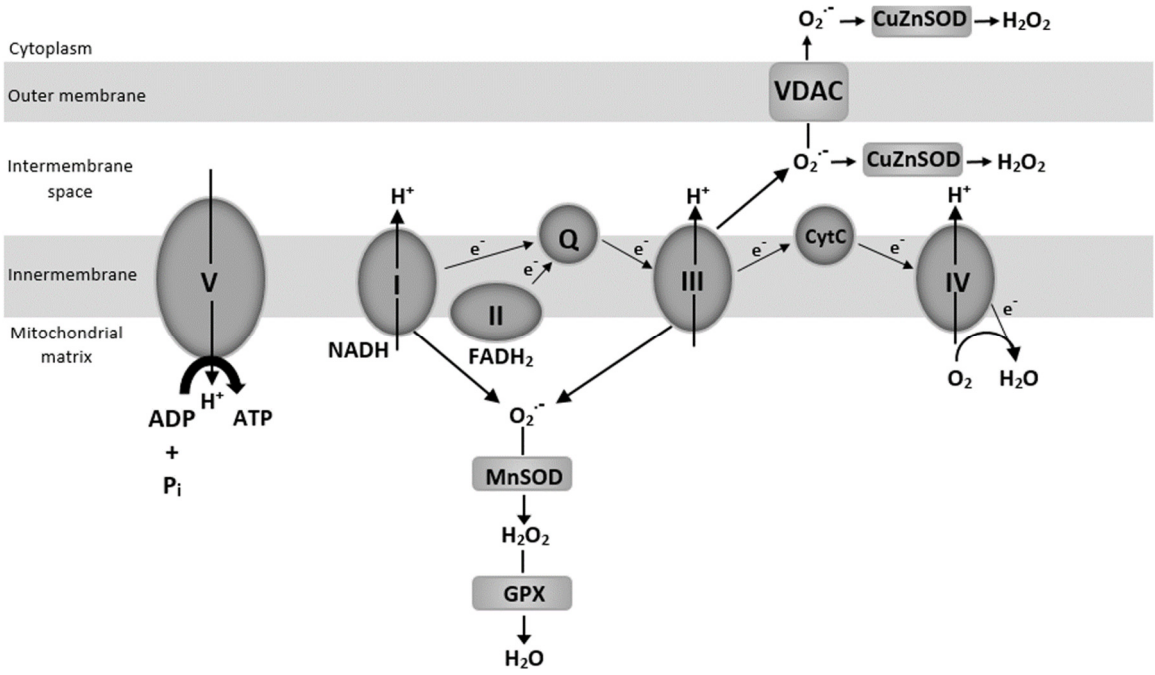

Fig. 2 Schematic drawing of the mitochondrial oxidative phosphorylation system. Five complexes (CI-CV) in the mitochondrial inner membrane, together represent the OXPHOS system. NADH is oxidized by complex I and $FADH_2$ by CII, thereby releasing electrons that are transferred via electron carriers [ubiquinone (Q) and cytochrome c (CytC)] as well as complexes III and IV to the final acceptor, molecular oxygen. When electrons are transferred along the protein complexes, protons are pumped out through CI, CIII and CIV. The protons flow back through CV and drive the ATP-synthesis. Leakage of electrons from CI and CIII leads to production of O_2^-, that is released in the matrix and intermembrane space. O_2^- is also diffused via VDAC to the cytosol. Mn-SOD in the matrix and CuZn-SOD in the intermembrane space and the cytosol degrade O_2^- to H_2O_2. In the matrix H_2O_2 is further detoxified to water by GPX. Image modified from [157].

1.4.2 Oxidative stress and apoptosis

The role of the cells antioxidant systems are to protect the organism from excessive levels of ROS, i.e. oxidative stress, a condition when the production of ROS and the ability of the cell to defend against ROS are not in balance. Both the production and the detoxification of ROS can be disturbed [158, 159]. The production of ROS is disturbed, for example by defect mitochondria, since they release large amounts of ROS. The detoxification of ROS is attenuated in response to decreased antioxidative enzyme activities [160]. During this condition the levels of ROS increase to amounts that the cells cannot handle, and ROS becomes toxic to the cell. This condition, i.e. oxidative stress, contributes to several pathological states, such as inflammation, diabetes mellitus, tumor formation, mental disorders such as depression, and neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) [161-164]. ROS are considered a key factor in neurodegeneration by causing oxidative stress and cell death [165, 166]. However, ROS do not only cause oxidative stress but can also, at moderate levels, function as signaling molecules and directly control many brain functions. Neurons can sense, transmit and convert ROS signals into appropriate intracellular responses. In this way, ROS function as a food intake-regulating signaling molecule in the hypothalamus

affecting the Arc neurons and thereby regulate food intake and metabolism in response to metabolic signals. Increased levels of ROS inhibit food intake [167-169].

1.4.3 Complex I deficiency

CI is considered the main producer of ROS both during normal conditions, as a by-product of cellular metabolism, and during pathological processes such as neurodegeneration [170]. A disturbed CI could potentially result in decreased production of ATP [171], and accumulation of ROS [172, 173]. This may lead to apoptosis [174] and a start of many mitochondrial disorders. CI is also shown to the most sensitive of all OXPHOS complexes. [175, 176]. One study shows that CIII and CIV had to be inhibited by 70-80% before major changes in oxygen consumption was seen, while CI showed a marked reduction in oxygen consumption already at 25% of inhibition [177]. Thus, even a small defect in the function or assembly of CI can markedly increase the levels of ROS leading to oxidative stress, mitochondrial dysfunction and disease. Considering this, one can understand why CI deficiencies are the most common disorder of the OXPHOS system (OMIM: 252010).

With its 45 subunits, CI is the largest of all OXPHOS complexes [178-180]. These 45 subunits are encoded by mitochondrial or nuclear genes, and CI deficiencies can be caused by mutations in any of these genes or in genes encoding CI assembly factors [181-187]. CI deficiencies primarily affect tissues that require most energy, such as the brain and muscles. It usually starts from birth or affects children, and could commonly be a progressive neurodegenerative disorder. CI deficiencies have been associated with neurodegenerative diseases such as AD and PD as well as with several childhood neurodegenerative disorders. Here, the Leigh syndrome (LS), characterized by a rapidly progressive neurodegeneration linked to both neuronal loss and astrocytosis and with a normal onset during the first weeks or months after birth, is the most common disorder [188-193]. However, the clinical picture of CI deficiencies is highly diverse with varying symptoms; common symptoms include neurodegeneration, failure to thrive, growth retardation, poor feeding and muscle weakness [194]. Adding to that, CI dysfunction or mitochondrial dysfunction have in general been associated with several psychiatric disorders such as autism [195], bipolar disorder [196] and even AN [197].

1.4.4 Complex I assembly factors

The mutations causing CI deficiencies found in mitochondrial- or nuclear-encoded CI genes only give a genetic explanation for about 33% of the patients [198-200]. This indicates that CI assembly factors may have an important role in the development of the remaining CI deficiencies cases [185]. The failure to assemble a properly functioning CI has been shown to

be a great contributor to mitochondrial diseases [153]. The process of assembling mitochondrial CI involves, at different steps of the process, at least fourteen known assembly factors [180, 201-211] and three putative assembly factors [207, 212, 213]. Pathological mutations causing CI deficiencies have so far been found in ten CI assembly factors (Table 1).

Mutations in CI assembly factors cause diseases such as Leigh syndrome, leukodystrophy, cardiomyopathy, fatal lactic acidosis and encephalopathy, with Leigh syndrome being most common.

Table 1

Assembly factors of human complex I

GENE NAME	OTHER NAME	DISEASE CAUSING MUTATION FOUND	PUBLICATIONS
NDUFAF1	CIA30	X	[211, 214-216]
NDUFAF2	B17.2L	X	[199, 202, 217-221]
NDUFAF3	C3orf60	X	[206, 222]
NDUFAF4	C6orf66	X	[206, 222]
NDUFAF5	C20orf7	X	[214, 223-225]
NDUFAF6	C8orf38	X	[226]
NDUFAF7	C2orf56		[227]
ECSIT			[203]
ACAD9		X	[228-234]
TMEM126B		X	[209, 235]
NUBPL	IND1	X	[199, 236-239]
FOXRED1		X	[199, 240, 241]
TIMMDC1	C3orf1		[207]
TMEM70			[242]
COA1	C7orf44		[213]
TMEM186	C16orf51		[212]
ATP5SL			[207]

Putative assembly factors are in bold.

1.4.4.1 Ndufaf1

NADH dehydrogenase [ubiquinone] 1 alpha sub-complex assembly factor 1 (NDUFAF1) gene encodes a CI assembly factor protein for CI. Knocking down the *Ndufaf1* gene in a cell system results in 50% decrease of CI activity and 40% reduction of fully assembled CI, while overexpression of *Ndufaf1* gene leads to increased CI expression [201]. So far, there are three reports of disease-causing mutations found in the *Ndufaf1* gene. One of them reported that the mutation in the *Ndufaf1* gene results in markedly reduced CI activity in a patient that presented FTT at 11 months of age [214]. In the other two cases the *Ndufaf1* mutation caused hypertrophic cardiomyopathy or leukodystrophy, the latter a disorder characterized by degeneration of the white matter in the brain [215, 216].

1.4.5 Mitochondrial involvement in neurodegenerative diseases

Neurodegenerative disorders are characterized by a relatively selective neuronal cell death. Various neurodegenerative disorders affect different parts of the brain and therefore also show different symptoms. Neurons have a high-energy demand as well as a limited regenerative capacity, making them particularly sensitive to damage caused by oxidative stress [243]. That in combination with a mitochondrial dysfunction can lead to neuronal cell death, showing the importance of having 'intact' mitochondria for a healthy neuron. Damaged mitochondria can result in increased production of proapoptotic factors and ROS, changes that can lead to neuronal damage [159]. Indeed, several studies show impaired mitochondrial function, increased production of ROS and oxidative stress in many neurodegenerative diseases. In many studies, the impaired mitochondrial function is considered to be a cause rather than a consequence of the neurodegeneration [244-247].

1.4.5.1 Complex I Dysfunction and Parkinson's Disease

The first sign of a mitochondrial dysfunction in relation to a neurodegenerative disease was detected in PD [248]. The symptoms seen in PD patients are a consequence of the neurodegeneration and loss of dopamine (DA) neurons in the substantia nigra. The degeneration of these neurons has been correlated with CI dysfunction and increased oxidative damage [248-251]. The substantia nigra, as well as the cortex of PD patients show decreased CI activity [252]. PD brains show oxidatively damaged CI subunits, leading to misassembly and functional impairment of CI [250].

1.5 INSULIN AND THE REGULATION OF GLUCOSE HOMEOSTASIS

In the endocrine pancreas, we find β-cells producing insulin and α-cells producing glucagon. α-cells and β-cells are located in clusters called the islets of Langerhans. We also find other cells producing other hormones, such as delta cells secreting somatostatin and gamma cells secreting pancreatic polypeptide. β-cells sense the levels of blood sugar, and release insulin in response to elevated blood glucose concentration in order to reduce the high glucose concentration. The insulin secretion from the β-cells starts by the uptake of glucose via the type 2 glucose transporters (GLUT2). After the entry into the cell, glucose is phosphorylated by the enzyme glucokinase. Glucose is then further metabolized during the glycolysis to create ATP, resulting in an intracellular increase in the ATP/ADP ratio. This increase results in closure of the ATP-sensitive potassium channels, preventing the potassium ions from crossing the cell membrane. This in turn results in an increased concentration of potassium ions inside the cell and thereby a rise in the positive charge inside the cell, causing membrane depolarization. As a result of this, the voltage-gated Ca^{2+} channels transport calcium ions into the cell. The increase in intracellular calcium concentrations results in release of insulin from the β-cells into the blood (Fig. 3) [253, 254].

The role of the secreted insulin is to reduce the blood glucose concentration by promoting the absorption of glucose from the blood [71]. Insulin does this by stimulating absorption of glucose from the blood to supply tissues, such as skeletal muscles, with glucose for energy production. Insulin also stimulates various tissues to absorb and store unused, left over glucose: in the liver and muscles that store as glycogen or in the fat tissue that converts glucose to triglycerides for storage. This is done when insulin molecules bind to their insulin receptor, that in turn promotes the uptake of glucose into the various tissues through the type 4 glucose transporter (GLUT4).

Insulin has the important role to prevent too high blood glucose levels, that are dangerous to the body; however, it is also important to regulate the insulin levels in the body, since too much insulin can result in dangerously low glucose levels, i.e. hypoglycemia. The assignment of the enzyme insulinase, found in the liver and kidney, is to ensure that insulin levels are regulated, by breaking down insulin circulating in the blood.

The stored glucose, in the form of glycogen and triglycerides, is used as a backup fuel source, when blood glucose levels decrease. The assignment for the α-cell-secreted glucagon is to give access to the stored glucose during times, when blood glucose levels fall. This is done by glucagon binding to receptors in specific tissues, resulting in activation of the enzyme glycogen phosphorylase which hydrolyses the stored glycogen to glucose.

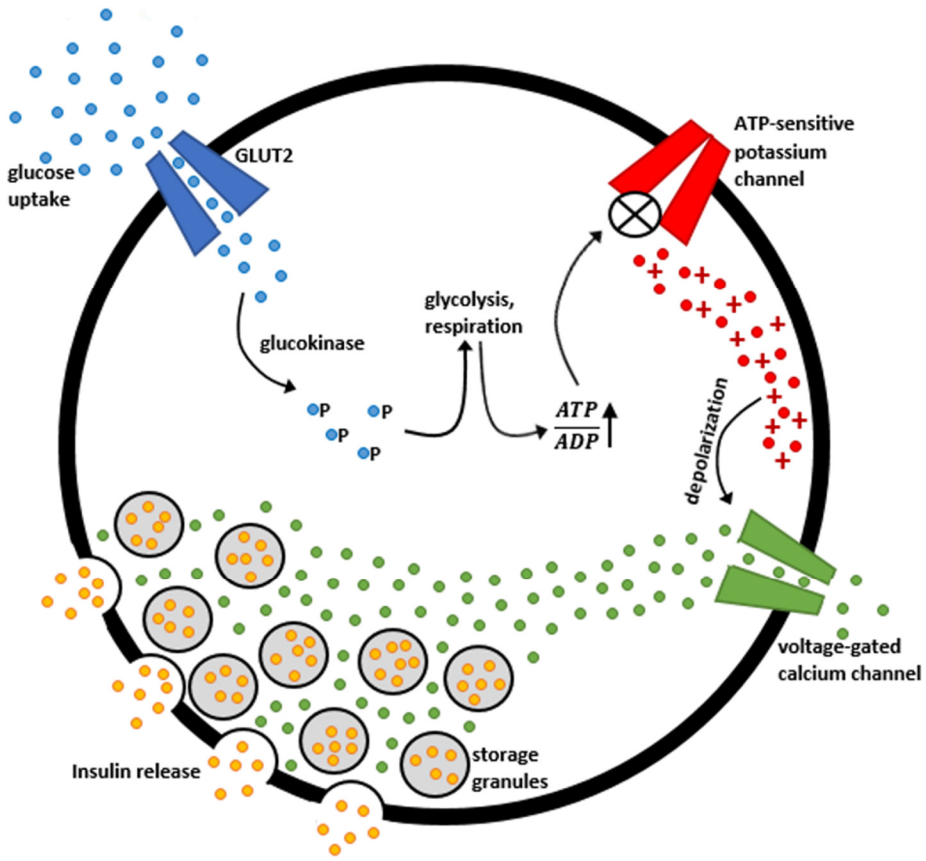

Fig. 3 Insulin secretion in β-cells is triggered by rising blood glucose levels. Starting with the uptake of glucose by the GLUT2 transporter, the glycolytic phosphorylation of glucose causes a rise in the ATP:ADP ratio. This rise inactivates the potassium channel that depolarizes the membrane, giving the inside of the cell a positive charge as shown by the red dots, causing the calcium channel to open allowing calcium ions to flow inward. The rise in levels of calcium leads to the exocytotic release of insulin from their storage granule. Image modified from Beta cell biology consortium 2004.

1.5.1 Insulin resistance

Insulin resistance is described as the inability of insulin to regulate the blood glucose levels in the body. This can lead to too much glucose circulating in the blood, a condition called hyperglycemia. Hyperglycemia can result in metabolic syndromes such as obesity and diabetes [255].

Diabetes mellitus is a metabolic disease and is characterized by abnormally elevated blood glucose levels, i.e. hyperglycemia. The hyperglycemia seen in diabetes mellitus is caused by either an inability of the body to produce insulin or an inability to produce insulin in sufficient amounts. In both cases the body cannot maintain the glucose homeostasis. There are two types of diabetes mellitus, the insulin dependent T1D and the insulin independent T2D. T1D is an autoimmune disease and is caused by destruction of the insulin-producing β-cells, resulting in hyperglycemia. T1D has an early onset. T2D is characterized by a slow continuous degradation of the glucose homeostasis as a result of insulin resistance and β-cell dysfunction, leading to

insufficient production and secretion of insulin. This will eventually result in a complete failure of the islet to secrete sufficient insulin to maintain glucose homeostasis. There are several risk factors associated with the development of T2D, some of which are disturbed eating habits, obesity and genetic predisposition [256].

Several factors have been shown to play a role in β-cell dysfunction and insulin resistance. Mitochondrial dysfunction, elevated levels of ROS and oxidative stress as well as hypothalamic inflammation are all implicated in pancreatic β-cell dysfunction. By providing ATP to support insulin secretion, mitochondrial oxidative phosphorylation is one of the important factors for a well-functioning insulin secretion, and an impaired mitochondrial oxidative phosphorylation has been associated with islet inflammation, β-cell dysfunction and the development of diabetes. In addition, mitochondrial dysfunction results in elevated levels of ROS and oxidative stress. Due to their low antioxidant capacity, β-cells are known to be extremely sensitive towards oxidative stress, and it is likely that oxidative stress induced by ROS is strongly involved in the impairment of β-cell function during the development of diabetes. Furthermore, oxidative stress is related to apoptosis, and loss of β-cell function caused by enhanced apoptosis is also an important factor in the development of diabetes [132, 257-261].

1.6 THE ANORECTIC *ANX/ANX* MOUSE

This thesis is based on a genetic animal model with a spontaneous mutation causing anorexia and starvation in mice. The anorexia (*anx*) mutation arose at Jackson laboratory in 1976 and is an autosomal recessive mutation characterized by poor appetite resulting in decreased food intake, reduced stomach content and body weight as well as starvation [31]. The *anx/anx* mouse develops growth failure and emaciated appearance, and dies at around 3 weeks of age, most likely because of the severe starvation [31]. No abnormal appearance is observed at birth, however, 5-8 days after birth the mice start to develop abnormal neurological behaviors, such as head weaving, body tremors, hyperactivity and uncoordinated gait. Experimental data have excluded all anatomical problems. Thus, no abnormalities in organs or in histology and anatomy of the gastrointestinal system have been found, and no biochemical defects are seen in the blood. The feeding pattern of these mice show a normal distribution, meaning that the daily changes in stomach content over time, are very similar between *anx/anx* and their normal wild-type (wt) littermates, indicating that the initiation of feeding is normal in the *anx/anx* mouse. However, at some point, around day 5 the *anx/anx* mice start to eat significantly less than their wt littermates, shown by the amount of food ingested, which then is not sufficient to sustain normal growth; this suggests a decreased appetite, indicating that the *anx/anx* mice cannot regulate the amount of food that they consume [31] (Fig. 4).

The anorexigenic neuropeptides POMC/CART and the orexigenic neuropeptides NPY/AgRP in the hypothalamus have been studied using both in situ hybridization (ISH) and immunohistochemistry (IHC). In Arc of wt mice, starvation is associated with an increase of the NPY and AgRP mRNA levels indicating activation of the orexigenic pathway, and a decrease of POMC and CART mRNA levels reflecting inhibition of the anorexigenic pathway. This represents a normal response to energy deficiency. In the anorectic *anx/anx* mice these systems do, however, show dramatic and aberrant expression patterns and levels in the hypothalamus [77, 262-271], even though somewhat different results have been reported in *anx/anx* mice. Some studies have shown NPY mRNA levels to be unchanged at P21 [266, 270], another one has shown an increase of both NPY and AgRP mRNAs [268]. IHC studies at P21 showed that AgRP and NPY neuropeptides are accumulated in the cell bodies in the Arc and that the density of NPY- and AgRP-positive fibers is reduced in all hypothalamic projection areas studied, e.g. PVN, LHA, DMH and Arc, when comparing *anx/anx* and wt mice [77, 263, 266, 268].

Alterations can also be seen in the POMC/CART neurons in the *anx/anx* Arc, including decreased mRNA levels of POMC and CART, as well as a reduced density of NPY receptor Y1-positive dendrites and cell bodies of the POMC neurons [262, 263]. An inhibition of these neurons is also seen during starvation in wt mice, indication a normal response to starvation. However, the decreased levels of mRNA for the NPY receptor Y1, which is a marker for POMC neurons, indicate that the POMC mRNA decrease could be a result of a decreased number of neurons. Also, using IHC, by P21, the *anx/anx* mice show fewer hypothalamic fibers immunoreactive (ir) for α-MSH, one of the POMC peptides, and CART [262, 263]. These results indicate a neurodegeneration in the anorexigenic POMC system of *anx/anx* mice [77].

When studying the serum leptin levels, it is shown that, from around postnatal day 8, the *anx/anx* mice show significantly reduced serum leptin levels, which is consistent with the low amounts of adipose tissue in *anx/anx* mice [262]. Reduced levels of leptin should increase the hunger signals, resulting in increased food intake. The fact that an opposite response to low leptin levels is seen, represents a further sign of a hypothalamic problem in feeding behavior.

In addition, the *anx/anx* mice show an increased density of serotonergic fibers in the Arc as well as in the olfactory bulb, frontal cortex, hippocampus and cerebellum [264, 270]. Serotonin has been shown to inhibit food intake, appetite, and to inhibit the activity of NPY neurons [272]. Other studies have also shown alterations in the dopaminergic [269] and the noradrenergic [271] systems in *anx/anx* mice.

Moreover, both inflammation and apoptosis have been related to the *anx/anx* mice, as shown in two microarray studies. The gene expression study by Lachuer et al. [273] showed hypothalamic inflammation in the *anx/anx* mouse and suggested that this hypothalamic inflammatory process could be the cause of the anorectic phenotype in the *anx/anx* mouse. The other study, by Mercader et al. [274], showed increased levels of deregulated genes involved in cell death and morphology. They also detected alterations in several signaling pathways for importance of energy homeostasis.

An earlier study on the *anx/anx* mouse showed increased levels in cell proliferation and apoptosis in dentate gyrus of the *anx/anx* mice [275]. The apoptotic protease activating factor 1 (APAF1) is another gene shown to be related to the phenotype of the *anx/anx* mice [276].

A higher expression of the neurotrophin receptor tyrosine kinase 3 (NTRK3) gene has been detected in the hypothalamus of the *anx/anx* mouse. Also, this gene shows a strong and significant association to eating disorders [277].

A recent study on the *anx/anx* mouse identified a mutation in the tyrosine receptor kinase 3 (Tyro3) gene and showed that Tyro3-transgenic *anx/anx* mouse doubled the weight and survival as well as expressed almost normal numbers of hypothalamic NPY neurons. However, since the specific mutation in the Tyro3 gene is also present in several other mouse strains, and none of these strains display any of the *anx* phenotypes, the Tyro3 variant are excluded as the *anx*-mutation [278].

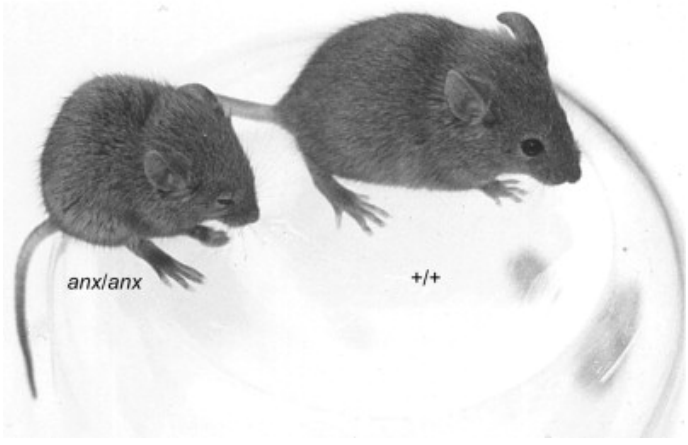

Fig. 4 Image showing an anorectic *anx/anx* mouse (to the left) and its normal wt littermate (to the right) at P21.

2 AIMS OF THE STUDY

The overall goal of this thesis is to increase the knowledge about the molecular mechanisms involved in energy homeostasis, i.e. the regulation of food intake and energy expenditure, in particular the role of (neuro)inflammation and mitochondrial dysfunction.

Specific aims are:

1. To assess the role of hypothalamic neuroinflammation on the regulation of food intake in an anorectic condition (**Paper I-II**).

2. To assess the role of mitochondrial dysfunction on the regulation of food intake in an anorectic condition (**Paper III** and **V**).

3. To assess the role of pancreatic inflammation on glucose homeostasis and ß-cell function in an anorectic condition (**Paper IV**)

3 MATERIALS & METHODS

3.1 ANIMALS (PAPER I-V)

All animal experiments performed in this thesis were approved by the local ethical committee, Stockholm Norra Djurförsöksetiska Nämnd. Originally the heterozygous *anx* breeding pairs (B6C3Fe-a/a-anx A/+ a) were obtained from The Jackson Laboratory [31]. The breeding pairs were used to set up an intercross. All pups (*anx/anx*, +/+, *anx*/+) were housed in ventilated cages at room temperature (22°C) with a 12-h light-dark cycle and had full access to food and water, as well as unlimited access to their mother. Genotyping was performed using simple sequence-length polymorphism (SSLP) markers mapped to the sub-chromosomal region, where the *anx* mutation is located [31].

3.2 MAPPING OF THE ANX-GENE (PAPER III)

The *anx*-interval had previously been mapped to a 20cM interval on mouse chromosome 2 [31]. To further narrow down the *anx*-interval two intercrosses were set up. Cross 1 was set up between (B6C3Fe-anx A/+ a × B6C3H F1) and resulted in 2050 F2 progeny (4100 meioses). Cross 2 was set up between (B6C3Fe-anx A/+ a and CAST/Ei) and resulted in 372 F2 progeny (744 meioses), and the results from this cross were analyzed. DNA was prepared from the tail tips. SSLP markers mapped to this subchromosomal region were used to genotype all mice. The previously reported chromosomal map position linked to chromosome 2 was the basis for choosing the D2Mit markers. The additional markers, i.e. the Jojo primer pairs, were designed around simple sequence repeats: Jojo5 spans a GT-repeat and Jojo8 a CA-repeat, located in the *anx*-interval.

3.3 IN SITU HYBRIDISATION (PAPER II)

ISH is a method used to study cellular expression and localization of mRNA in specific tissues and cells. The ISH used in this thesis was based on radioactively labelled oligoprobes complementary to 48 nucleotide bases, and was used to study the expression of MHC class I related genes in *anx/anx* and +/+ mice.

Brain tissue from *anx/anx* and +/+ mice were frozen on dry ice and cut in thin sections using a cryostat. Immediately after cutting, the brain sections were mounted on glass slides. The anti-sense oligoprobes for MHC Class I were synthesized by Cybergene AB (Huddinge, Sweden). The isotopic-labeling of the oligoprobes at the 3 end was done enzymatically. The labeled probes were hybridized to the brain sections by emerging the slides in a cocktail including the oligoprobes after which the slides were washed, air-dried, dipped in NTB2 nuclear emulsion

(Kodak, Rochester, NY), exposed, developed and then analyzed a microscope equipped with a dark-field condenser and a digital camera.

The validity of the results obtained with ISH is dependent on the specificity of the probe. To test the probe specificity, we incubated the slides in a mixture including both labeled probe and an excess of unlabeled probe, a procedure that always resulted in loss of signal.

3.4 HISTOCHEMISTRY (PAPER I, II, III)

IHC is a method used to detect expression of specific proteins/peptides and their cellular localization in different tissues. Also, using double or triple-labeling with several markers a co-localization of different proteins can be studied.

In **Paper I, II** and **III** we used IHC to detect expression of AgRP, TLR2, ionized calcium binding adapter 1 (Iba1), a marker for activated microglia cells, MHC Class I subunit β2 microglobulin (β2m), active caspase 6 (actCasp6), an enzyme involved in axonal degeneration, the Y1R and SOD2 in *anx/anx* and +/+ hypothalamus. In **Paper IV** we used IHC to study pancreatic and cultured islets to detect expression of Iba1, F4/80, a marker for macrophages, the pancreatic hormones; insulin, glucagon and somatostatin, and Ki-67, a marker for proliferation.

For IHC analysis, mice were anesthetized with isoflurane and perfused via the ascending aorta with Tyrode's Ca^{2+}-free solution, followed by a mixture of paraformaldehyde and picric acid. Brains and/or pancreas were then rapidly dissected and immersed in the same fixative for 90 min followed by overnight incubation in sucrose solution. Sections were cut on a cryostat, thaw-mounted on to slides and then further processed for either indirect IHC technique or the tyramide signal amplification (TSA)-plus system. From this point the sections were used for TUNEL labeling as described in section 3.4.1.

In the indirect method, we first incubated overnight with a primary antiserum, directed against our target of interest, followed the next day by incubation with secondary antibodies, directed against the species that primary antisera were raised in and conjugated with a fluorescent marker.

When using the TSA-plus system, we first incubated with primary antisera overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies and then with biotinyl tyramide. The function of the HRP-conjugated antibody is that HRP enzyme catalyzes the deposition of several activated tyramide molecules. The advantage is that the additional step in the TSA-plus system amplifies the signal.

After IHC with either technique, the samples were analyzed using a fluorescence microscope and/or a confocal scanning microscope.

3.4.1 TUNEL Labeling (Paper II)

Terminal dUTP Nick End Labeling (TUNEL), a marker for cell death, was used to investigate the number of apoptotic cells in the *anx/anx* hypothalamus. TUNEL labeling is a method used to detect DNA fragmentations, which results from apoptotic signaling cascades. This is done by enzymatical labeling of the terminal end of the nucleic acids in apoptotic cells by terminal transferase [279].

For TUNEL labeling, the procedure is the same as for IHC up till the sections are thaw-mounted on to slides. The sections were then pretreated with H_2O_2 in methanol to block endogenous peroxidases. Following this step, a permeabilization solution was applied to the sections before the TUNEL reaction mixture was added. Sections were then washed, mounted and finally analyzed in a fluorescence microscope.

3.4.2 Dihydroethidium injections (Paper III)

To evaluate whether increased production of ROS occurs in the hypothalamus of *anx/anx* mice we performed dihydroethidium (DHE) injections. DHE is oxidized to fluorescent ethidium when reacting with superoxide [280]. To measure hypothalamic ROS production an intravenous DHE injection was given once via the femoral vein to *anx/anx* and wt mice at P21. Two hours after injections the mice were deeply anaesthetized and sacrificed by perfusion with fixative (as in 3.4). Sections were cut on a cryostat and processed according to indirect IHC methods to visualize the AgRP and NPY arcuate neurons and microglia. Red fluorescent dot-like structures in the Arc were counted in *anx/anx* and +/+ mice.

3.4.3 Pancreatic and cultured islets (Paper IV)

We performed IHC to study inflammation in intact pancreas and in isolated islets cultured overnight using Iba1 and F4/80, which both are markers for macrophages/microglia. Immunohistochemically double-labeling was done with antiserum to Iba1 and insulin, or Iba1 and F4/80. We also analyzed the pancreatic hormones glucagon and somatostatin, and Ki-67, the latter a marker for proliferation.

In order to study intact pancreas, mice were anesthetized with isoflurane, perfused with formalin fixative and then further processed for IHC as described above. Sections were incubated with antiserum against Iba1, F4/80, glucagon, somatostatin or Ki-67 overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody, and

processed with the TSA-plus fluorescein system. For double-labeling, the TSA was followed by incubation with antibodies against insulin or Iba1, and another incubation using secondary antibodies conjugated with Cy3 or rhodamine red. Finally, the sections were counterstained with 4,6-diamidino-2phenylindole (DAPI), a blue fluorescent stain binding to AT regions of DNA.

For IHC on isolated islets cultures, the isolation of the islets was performed as described in section 3.12. After culturing the islets overnight, they were fixed for 20 min in ice-cold paraformaldehyde and picric acid fixative, rinsed in phosphate-buffered saline and frozen on dry ice. The islets were then cut on cryostat and thaw-mounted on to slides. IHC was performed as described above (3.4).

3.5 MICROARRAY (PAPER III)

Microarray expression analysis is a method used to evaluate differences in gene expression in a large set of genes. We used Affymetrix Mouse Genome 430 2.0 Array (Affymetrix Inc.) to detect differences in expression of genes in the Arc between *anx/anx* and +/+ mice at P21. RNeasy kit (Qiagen) was used to prepare total RNA from the Arc using 3 *anx/anx* and 3 +/+ mice. cRNA was synthesized from total RNA and then hybridized to the Array. MAS 5.0 software, was used to analyze the data files. The expression values were determined by the MAS 5.0 software, and the genes were included for comparison analysis, if expression was present in at least four out of six arrays. After that we compared all *anx/anx* and all +/+ samples, giving a total of nine comparisons, and the genes were included for pathway analysis, if the expression was altered in at least seven out of the nine comparisons.

3.5.1 Ingenuity Pathway Analysis (Paper III)

The microarray expression analysis identified a large set of genes with altered expression in the Arc comparing *anx/anx* and +/+ mice. To identify the top canonical pathways involved in the phenotype of the *anx/anx* mice, these genes were used to perform Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com). Genes with a fold change ≥1.4 increase or decrease were included, giving 132 up- and 73 down-regulated genes in the IPA analysis. All pathways included had a P-value < 0.05.

3.6 REAL-TIME PCR (PAPER III)

Real-time PCR was used to analyze the expression levels of the candidate gene for the candidate *anx*-mutation, *Ndufaf1,* in several tissues. The tissues analyzed were brain, heart, kidney, lung, pancreas, liver and skeletal muscle. Total RNA for both *anx/anx* and wt mice at

P21 was prepared from these seven different tissues, where after cDNA was synthesized from total RNA by reverse-transcription. The samples were then analyzed for the expression of *Ndufaf1* using Taqman real-time PCR (7900 HT Fast Real-time PCR system, Applied Biosystems Inc., Foster City, CA). The amplification of the genes was performed in duplicates. The calculation of relative gene expression was done using the standard curve method. Quantity was calculated using mean values of the duplicates relative to those for well-known house-keeping genes, with a specific house-keeping gene chosen for each specific tissue.

3.6.1 Mitochondria count (Paper III)

A mitochondrial count was performed with SYBR green real-time PCR to make sure that the defects in the OXPHOS system seen in the *anx/anx* mice were not due to a reduced number of mitochondria. DNA from four *anx/anx* and four +/+ hypothalamus were prepared using DNeasy Qiagen (Qiagen, Washington DC, Maryland). Probes directed against markers for mitochondrial DNA (cytochrome B and cox1) and nuclear DNA (cyclophyllin) were used to calculate the proportions of mitochondrial DNA/genomic DNA in hypothalamus from *anx/anx* and +/+ mice. To quantify the number of mitochondria the Sequence detector system ABI-prism 7000 (Applied Biosystems Inc.) was used.

3.7 SDS PAGE (PAPER III, V)

Sodium dodecyl sulfate – polyacrylamide gels (SDS-PAGE) is a method used to separate proteins according to their molecular weight. We used SDS-PAGE to analyze protein levels of NDUFAF1, AMPK and glucose transporters (GLUTs) in total brain and hypothalamic tissue.

The tissue was homogenized, centrifuged and boiled, where after the protein concentration was determined using BCA Protein Assay kit (Pierce, Thermo Scientific). The proteins were separated on a gel and were then transferred to a membrane, and detected by immunoblotting using primary antibody against the protein of interest. Secondary antibodies conjugated with horseradish peroxidase were then applied, the signal developed, exposed to film and the intensities of the bands were determined with Image J or MultiGauge (Fujifilm, Tokyo, Japan). Data were normalized to the expression of β-actin.

3.8 BLUE NATIVE PAGE (PAPER III)

Blue Native PAGE (BN PAGE) was used to determine the amount of fully assembled mitochondrial protein CI and sub-complexes in the OXPHOS system. BN PAGE is a non-denaturating blot, which means that it does not separate the protein complexes, but keeps them together as they are in the cell cytoplasm. Hypothalamus was dissected form *anx/anx* and wt

mice at P21, whereafter crude hypothalamic mitochondria were isolated as previously described [281]. BCA Protein Assay kit (Pierce, Thermo Scientific) was used to determine mitochondrial protein concentration and NativePAGE™ Sample Prep Kit (Invitrogen) for mitochondrial protein preparation, where after BN-PAGE was performed. The mitochondrial proteins were run on NativePAGE™ gradient gels (Invitrogen). During the electrophoresis with BN-PAGE coomassie blue dye is used, giving the complexes a negative charge allowing separation. After electrophoresis, gels were further processed for SDS PAGE to detect CI, CII and TOM40, used for normalization, or for in-gel activity staining of CI (see 3.8.1).

3.8.1 In- Gel Activity Assay (Paper III, IV)

In-gel activity staining was used for the measurement of CI activity in the hypothalamus and in crude mitochondria from *anx/anx* and wt islets. Immediately after BN-PAGE gel electrophoresis, the gels were used for CI in-gel activity staining. The gel was immersed in a solution containing Tris-HCl, NADH and Nitro Blue tetrazolium (NBT). The intensities of the bands were analyzed using Image Gauge software. Data were normalized to the expression of TOM40, which is a marker for mitochondrial outer membrane.

3.9 SEQUENCING (PAPER III)

Sequencing of the candidate gene *Ndufaf1* was done to identify sequence alterations between *anx/anx* and +/+ mice. Genomic DNA and cDNA were prepared, followed by PCR amplification and sequencing using ABI 3730 DNA Analyzer and BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc, Foster City, CA). In brief, genomic DNA was prepared using mouse tail tips and standard salting-out procedures. RNA/cDNA was prepared using total brain, and RNA was extracted using TRIZOL Reagent. After DNase treatment of the RNA (TURBO DNA-free; Ambion), cDNA was synthesized by reverse-transcription of total RNA with oligoDT (SuperScript III First Strand Synthesis System for RT-PCR; Invitrogen).

3.10 ALLELE SPECIFICITY (PAPER III)

Allele specificity analysis was performed to evaluate whether the downregulation in *Ndufaf1* is related to the mutation rather than a secondary effect of the starvation. Upon sequencing the *Ndufaf1* gene a silent T/C SNP was found (Ensembl gene ID ENSMUSG00000027305 *Ndufaf1* +87 T/C). This SNP was used to separate the expression level of the *anx*-allele from the wt-allele. Total brain from four heterozygous (*anx*/+) mice was used, since these mice carry one copy of each allele and show no disease phenotype. RNA and DNA were isolated using TRIZOL, and cDNA was prepared in triplicates from the RNA. Duplicates of the DNA and

cDNA were PCR-amplified over the T/C SNP, where after the levels of the allele expression were quantified using PyroSequencing (PSQ 96; Qiagen). Imbalanced expression levels of the two alleles were obtained by comparing the ratio of the mean peak heights from the two alleles in cDNA to the corresponding peak heights in genomic DNA [peak height *anx* allele (cDNA)/peak height wt allele (cDNA)]/[peak height *anx* allele (gDNA)/peak height wt allele (gDNA)].

3.11 RESPIROMETRY (PAPER III)

The efficiency of the different complexes in the OXPHOS system was studied in the *anx/anx* hypothalamus by high-resolution respirometry utilizing an Oxygraph-2K (Oroboros, Innsbruck, Austria). Using this device one can evaluate the rate of respiration by measuring oxygen consumption. Hypothalamus was dissected and homogenized in a buffer named MIR05 including EGTA, $MgCl_2$, KH_2PO_4, HEPES, sucrose, taurine, BSA and K-lactobionate. The hypothalamic tissue suspension was added to the Oxygraph-2K chamber. After saturating the respiration medium with oxygen, substrates such as glutamate and ADP were added to measure different states of respiration.

First, basal oxygen flux (leak) from CI was assessed by adding the CI substrates malate and pyruvate. In order to couple electron transfer to ATP production, i.e. to measure the coupled respiration from CI, ADP and glutamate were added. The CII substrate succinate was added to assess oxygen flux from CI and CII. Maximal capacity of the electron transfer system by CI and CII was assessed by adding the uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). Electron transport through CI was then inhibited by adding rotenone, and through CIII by antimycin A. After inhibiting CI and CIII we assessed the remaining oxygen flux, i.e. the oxygen flux independent of the electron transfer system, which was subtracted from each of the previous steps.

3.12 PANCREATIC ISLET ISOLATION (PAPER IV)

Isolated pancreatic islets were processed for several analyzes, e.g. of glucose homeostasis, static and dynamic insulin secretion, and for IHC to study markers of inflammation. For isolation of pancreatic islets from *anx/anx* and wt mice, the collagenase method was used that allows preservation of islets [282]. Pancreas was inflated with Hank's balanced salt solution containing collagenase. After digestion, islets were picked manually under a microscope and cultured overnight in RPMI-1640 medium containing glucose.

3.13 PANCREATIC ISLET SIZE DISTRIBUTION (PAPER IV)

The size of the pancreatic isolated islets from *anx/anx* and wt mice was determined and measured on micrographs using 40-100 islets/animal.

3.14 ELISA (PAPER IV)

Enzyme-linked immunosorbent assay (ELISA) is a method to identify the presence of different substances by using antibodies and color changes. An antibody linked to an enzyme binds to the specific antigen of interest. In the final step a substance containing the enzyme substrate is added, resulting in a reaction that produces a detectable signal, e.g. a change in color in the substrate. We used ELISA to measure insulin concentration.

3.14.1 Intraperitoneal Glucose Tolerance Test (Paper IV)

To analyze glucose homeostasis in *anx/anx* and wt mice, we used the intraperitoneal glucose tolerance test (ipGTT) in both fed state and after 5 h of fasting. Glucose and insulin values were measured in blood samples taken from the tail tip. The time points measured were 0, 30, 60 and 90 min after the mice received a glucose injection. 0 min represents the basal state and was taken immediately before the mice received the glucose injection. An ip injection containing 2 mg glucose/g body weight was given. Glucose levels were measured throughout the test using an Accu-Chek blood glucose analyzer (Roche Diagnostics, Indianapolis, IN). For insulin measurements, serum was prepared from the blood samples, and insulin levels were measured using Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden).

3.14.2 Total pancreatic insulin and glucagon content (Paper IV)

We analyzed the pancreatic hormones, insulin and glucagon in pancreas. For the measurement of total insulin and total glucagon content, pancreatic hormones were extracted from whole pancreas with acid ethanol, where after the extracts were analyzed using Ultrasensitive Mouse Insulin ELISA or Glucagon ELISA (Mercodia). Total protein content was measured in the extracts with BCA Protein Assay kit (Thermo Scientific) and was used for normalization.

3.14.3 Static insulin release (Paper IV)

Static insulin release was measured in isolated *anx/anx* and wt islets by stimulation with 3 mM glucose, 11 mM glucose and 25 mM KCl. The stimulation with both glucose and KCl enabled the measurement of insulin release at different stages of the insulin-release-pathway. Islets were isolated as described above, and groups of 3 islets/genotype were first preincubated in Krebs-Ringer bicarbonate buffer (KRBB) containing 3 mM glucose. The islets were then incubated in the same buffer containing low glucose or high glucose concentration or KCl. The

media were analyzed for insulin levels using Ultrasensitive Mouse Insulin ELISA kit (Mercodia), and the values were normalized by the number of islets.

3.14.4 Free fatty acids in serum (Paper IV)

We evaluated the levels of free fatty acids (FFAs) in serum from nonfasted *anx/anx* and wt mice. This was done by the use of Free Fatty Acid Quantification kit (Sigma-Aldrich). The concentration of FFAs is determined by a coupled enzyme assay, resulting in a fluorometric product which is proportional to the FFAs concentration in the serum analyzed.

3.15 ARCDIA TPX ASSAY (PAPER IV)

The ArcDia TPX technology (Arc-Dia Group, Turku, Finland) was used for the measurement of insulin concentrations in pancreatic islets from *anx/anx* and wt mice. This system detects the signal of a fluorescent labeled antibody by two-photon excitation from the surface of each individual microsphere. The technique uses the antibody-antigen interaction, and an immunecomplex consisting of monoclonal antibody, antigen and fluorescent labelled monoclonal antibody is formed in proportion to the analyte concentration. Both the bound tracer, i.e. the specific signal, and the unbound tracer, i.e. the background signals, are recorded. The levels of insulin were obtained with the help of human insulin standard curve using samples of known concentration.

3.15.1 Dynamic insulin release (Paper IV)

In addition to the static insulin measurement with ArcDia in isolated pancreatic islets, dynamic insulin release was also monitored. This allows for insulin measurement over time analyzed during continually perfused islets at 3mM basal glucose concentration or stimulated with 11 mM glucose or 25 mM KCl. For measurement of dynamic insulin release, islets were isolated as described above, and 30 islets/genotype were preincubated in KRBB containing 3 mM glucose. The islets were immobilized and continuously perfused, and stimulated with 3 mM glucose followed by 11 mM glucose, 3 mM glucose and lastly 25 mM KCl. Samples were collected every second minute, and insulin was measured in using the ArcDia TPX assay (Arc-Dia Group).

3.15.2 Islet insulin content (Paper IV)

We measured the insulin content in isolated islets. For this, isolated islets from the two different genotypes were used. The islets were incubated with M-PER for and then centrifuged. The supernatant was used to analyze insulin levels with ArcDia TPX assay (Arc-Dia Group). For total protein content, islets were homogenized in RIPA buffer, and total protein content was

measured in the extracts using BCA Protein Assay kit (Thermo Scientific). Total insulin content in *anx/anx* and wt pancreas was normalized against total pancreatic protein content.

3.16 SPECTROPHOTOMETRIC ANALYSIS

Spectrophotometric analysis was used to measure cytoplasmic free Ca^{2+} concentration and mitochondrial membrane potential in isolated islets, as well as respiratory chain enzyme activities in the hypothalamus from *anx/anx* and wt mice.

The spectrophotometric technique uses the attributes of light to measure concentration of different compounds in a solution. Different compounds having different atomic and molecular interactions have different absorption spectra. The spectrophotometer can measure the amount of light absorbed by the solution by measuring the intensity of a light beam after passing through a solution.

3.16.1 Measurement of $[Ca^{2+}]_i$ (Paper IV)

We measured changes in free cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$,when islets from *anx/anx* and wt mice were stimulated with high glucose concentration. For the correct measurement of intracellular calcium concentration, we used fura 2 acetoxymethyl (AM), a fluorescent indicator dye for measuring intracellular calcium. Changes in $[Ca^{2+}]_i$ were measured as the 340/380 nm fura-2 fluorescence ratio. Islets isolated from *anx/anx* and wt mice were incubated for 50 min at 37°C in KRBB containing 3 mM glucose and fura 2 AM. A single islet was transferred to a chamber and fixed to the cover slip with PuraMatrix Peptide Hydrogel. The chamber was mounted on a microscope connected to a Apex Fluorolog spectrophotometer. Changes in $[Ca^{2+}]_i$ were measured over a period of time as the islets were continuously stimulated with 11 mM glucose, followed by 3 mM glucose and finally 25 mM KCl.

3.16.2 Mitochondrial membrane potential of pancreatic islets (Paper IV)

Mitochondrial membrane potential (MMP) was measured in isolated islets from *anx/anx* and wt mice. This was done using Rhod123, a fluorescent dye. This dye has a negative charge and will therefore strive for an equilibrium across the membrane. The negative charged dye will accumulate in the mitochondrial membrane matrix space in inverse proportion to the MMP, so that a more negative (i.e. more polarized) MMP will accumulate more dye. The total force that drives the protons back into the mitochondria (i.e. Δp) to generate ATP is a combination of the MMP, which is the charge gradient, and the mitochondrial H^+ concentration gradient. The specific measurement, using this method, is the charge gradient across the inner mitochondrial membrane and is not specifically a measurement of the mitochondrial proton gradient Δp.

Isolated islets from *anx/anx* and wt mice were incubated in KRBB with 3 mM glucose and Rhod123 for 10 min. The increased MMP, in response to the change from 3 to 11 mM glucose, was recorded using the same system as for the $[Ca^{2+}]_i$ measurements.

3.16.3 Respiratory chain enzyme activities in hypothalamus (Paper V)

We also used spectrophotometric analysis to evaluate the enzymatic activity of the different complexes in the respiratory chain in isolated mitochondria from hypothalamus of *anx/anx* and +/+ mice.

In addition, we spectrophotometrically evaluated citrate synthase (CS) activity in hypothalamic tissue. CS is a mitochondrial marker [283], and was used for quantitative analysis of the number of mitochondria in *anx/anx* vs wt hypothalamus. Hypothalamus was dissected and homogenized in a solution with Triton X-100, KH2PO4 and EDTA. The homogenate was centrifuged and CS activity determined in the supernatant. The assay was performed on a photometer.

3.17 EX VIVO AUTORADIOGRAPHY OF HYPOTHALAMIC GLUCOSE UPTAKE (PAPER V)

To analyze the degree of hypothalamic metabolism, the rate of glucose uptake in *anx/anx* hypothalamus was studied by using intra peritoneal (ip) injection of 2-deoxyglucose labeled with tritium, also called hydrogen-3 [^{3}H], a radioactive isotope of hydrogen. [^{3}H]-2-deoxyglucose is a good marker for tissue glucose uptake, and the regional distribution can be assessed by tissue-sectioning followed by autoradiography.

Prior to the ip injection of [^{3}H]-2-deoxyglucose the mice were fasted for 5-6 hours. Brain and liver were dissected forty-five min after injection, which is a sufficient time for adequate glucose uptake [284]. Tissues were sectioned and exposed to phosphor imaging plates, which were analyzed using Multi Gauge 3.2 phosphorimager software (Fujifilm). The glucose uptake into the hypothalamus, motor cortex, thalamus and liver was calculated by the following formula: (µmol glucose per mL serum/dpm per mL serum) × dpm/g tissue = µmol glucose/45 min/g tissue).

Also, by the time of killing, blood glucose concentration was measured using an Accu-Chek blood glucose analyzer (Roche Diagnostics). To estimate glucose-specific activity in serum, the serum glucose values were calculated by multiplying the blood values by 1.15. The radioactivity in serum was measured with a scintillation counter.

3.18 ANALYSIS OF METABOLITES & ATP TURNOVER (PAPER V)

Metabolites in the *anx/anx* hypothalamus were measured in the basal state and after one minute of ischemia. Phosphocreatine (PCr), creatine (Cr), ATP, Lactate, glucose 6-P (G6P) were measured with enzymatic techniques (changes in NAD(P)H) adapted for fluorometry. ATP, ADP, AMP and inosine monophosphate (IMP) were measured using by high-performance liquid chromatography (HPLC).

For the study of the basal condition, the whole head was decapitated, immediately frozen in liquid nitrogen (N_2) and freeze-dried, and the hypothalamus dissected out. For the ischemic/stress experiment, the hypothalamus was rapidly dissected out after decapitation, and frozen in liquid N_2 and freeze-dried. The process from decapitation to freezing in N_2 took approximately 1 min. Ice-cold perchloric acid was added to the freeze-dried tissue, that was kept in an ice bath for 20 min and then centrifuged. The supernatant was neutralized with 2.2M KHCO3 and centrifuged again. The latter supernatant was used to analyze metabolites with enzymatic techniques or with HPLC. Metabolite values were divided with the value for total Cr (PCr + Cr) and then multiplied by the mean total Cr content for the whole material. This was done to adjust for non-hypothalamic tissue (e.g. blood).

Anaerobic ATP turnover was calculated by using the following equation: $-2\Delta ATP - \Delta PCr + (1.5\Delta$ lactate). Δ refers to the mean difference between ischemic and basal values.

4 RESULTS & DISCUSSION

4.1 IMPAIRED DEVELOPMENT OF THE NPY/AGRP SYSTEM IN THE *ANX/ANX* MOUSE (PAPER I & II)

In **Paper I**, we studied the development of the NPY/AgRP system in *anx/anx* and +/+ mice by examining the AgRP distribution at different postnatal days. AgRP was used as a marker for the orexigenic system, since it is expressed only in the arcuate NPY neurons [63, 65, 77], whereas NPY is also expressed in many other brain systems [59]. AgRP distribution was studied at postnatal days P1, P5, P10, P12, P15 and P21 using IHC.

The development of the AgRP system is similar in the *anx/anx* and +/+ mice until P12. However, from this day the normal gradual increase in AgRP fiber density ceases, with a much weaker expression at P21 (the longest time studied). When comparing P21 and P15 the AgRP fiber density in *anx/anx* mice was significantly reduced and even disappeared in certain regions. At P21 we could also detect many strongly AgRP-positive cell bodies in the Arc in the *anx/anx* mice compared to +/+ mice, where only few and weakly fluorescent AgRP-positive cell bodies were observed.

In **Paper II** we also studied the postnatal development of the POMC/CART system in the *anx/anx* and +/+ mice using IHC. This was done using antibodies directed against the NPY receptor Y1, since this receptor is expressed by POMC neurons and decorates their soma and the dendrites. We could see that the POMC/CART system develops similar to the AgRP neurons until P21, although no changes were seen at P15. At P21, however, there was a decrease in density of the dendritic processes and cell bodies of POMC neurons in the Arc *anx/anx* mice. This shows that the abnormal pattern of the POMC/CART system in *anx/anx* mice can only be detected later in the postnatal development compared to the changes in the NPY/AgRP system.

Taken together, these results are indicative of degenerative processes in the hypothalamic Arc in the *anx/anx* mice, whereby the NPY/AgRP system is first affected and the POMC/CART system at a later stage.

4.2 SIGNS OF INFLAMMATION IN THE HYPOTHALAMUS OF THE *ANX/ANX* MOUSE (PAPER I & PAPER II)

4.2.1 Activated microglia and TLR2

A second aim of **Paper I** was to study the underlying mechanisms for the impaired development of the AgRP system in *anx/anx* mice. One of our hypotheses was that the

alterations in the development of this system could be associated with an inflammatory process. We therefore studied the expression of Iba1, which is a marker for activated microglia [285] as well as TLR2. Microglia and TLR2 are both a part of the innate immune system. Microglia cells are activated in the presence of damaged cells and different injuries in the nervous system, such as virus infection or degeneration [286, 287]. TLR2 is a protein receptor that defends against invading pathogens and toxic cell debris, e.g. apoptotic cells [288]. Iba1 and TLR2 were studied using IHC at the same time points as was used to explore AgRP and Y1 described above, e.g. postnatal days P1, P5, P10, P12, P15, P21 in *anx/anx* and +/+ mice.

Our results show that the gradual decrease in AgRP fiber density starting from P12 overlaps with an increase in Iba1 and TLR2 immunoreactivities (irs). Starting from P12 the detection of activated microglia (i.e. increased Iba1 labeling) was seen in several hypothalamic and extra-hypothalamic areas with a gradually stronger labeling at P15 and P21. This gradual increase in microglia activation was seen in the same areas as the decrease in density of AgRP immunoreactive fibers. The impaired development of the AgRP system and the overlapping activation of microglia of the *anx/anx* mouse indicate an inflammatory and/or degenerative process.

In **Paper I**, we could also see presence of activated microglia cells close to the NPY neurons in the Arc. This was further studied in **Paper II**, using IHC and antibodies against NPY, Iba1 and OX42, an additional marker for activated microglia. By double-labeling for either NPY and Iba1 or NPY and OX42 we saw that several activated microglia were tightly surrounding NPY/AgRP neurons. This pattern was similar to that described by Ribak et al. as a microglia-associated type of cell death which differs from both apoptosis and necrosis [289].

4.3 SIGNS OF NEURODEGENERATION OF THE ARCUATE NEURONS IN THE *ANX/ANX* MOUSE (PAPER II)

In **Paper II**, several approaches were taken to further investigate a possible neurodegeneration and inflammation in the *anx/anx* hypothalamus. As a result, we found additional evidence for neurodegeneration and show indications that both the anorexigenic and the orexigenic neuronal populations in the hypothalamus are likely to undergo degeneration in the *anx/anx* mouse.

First, we used IHC and ISH to study the expression and the localization of MHC class 1-related molecules. Expression of the MHC class I gene was found in Arc, both in neurons and in glia cells. We detected strong and region specific expression of MHC class I- related molecules in all areas that previously showed expression of activated microglia. That is in line with the

function of activated microglia, since they are well known to express MHC class I [124]. Glial cells express MHC class I during pathological conditions, such as multiple sclerosis, viral encephalitis and brain injury [123, 124]. However, we did not only see that the activated microglia cells expressed MHC class I, but also detected expression of the MHC class I co-subunit β2m by the NPY/AgRP and POMC/CART neurons in the Arc.

Neurons express MHC class I protein during acute inflammation and changes in electrical activity [119-122]. Both increased and decreased neuronal activity can result in increased MHC class I expression in the neurons [290, 291]. The expression of MHC class I is also important for the maintenance of synapses as well as for the regeneration after injury [292], indicating that MHC class I could be expressed to save the abnormally active neuron from synaptic elimination.

We therefore continued studying the activity of the hypothalamic neurons in *anx/anx* and +/+ mice by using two markers for cellular reactivity, cFOS and ΔFosB [293]. We could see that most of the β2m-expressing neurons in the Arc of the *anx/anx* mice had no or very low expression of Fos B. This indicates that the neurons expressing the MHC class I subunit are 'silenced' or have a very low activity.

In **Paper II**, we further analyzed the degenerative process in the hypothalamus of the *anx/anx* mice by using markers for cell death. The number of apoptotic cells was investigated using TUNEL labeling. We also used active actCasp6, which is an enzyme involved in axonal degeneration and has been shown to be increased in disorders such as Alzheimer's [294] and Huntington's disease [295].

At P21, a significantly increased number of apoptotic cells were found in several hypothalamic areas, such as Arc, VMH, LHA and DM, in the *anx/anx* compared to +/+ mice. Furthermore, at P21, we detected double-labeling of NPY and actCasp6 in Arc and DMH of *anx/anx* mice, further indicating degenerating NPY fibers. We could not see a clear detection of double-labeling of actCasp6 and α-MSH in *anx/anx* mice. Double-labeling of actCasp6 and NPY, or actCasp6 and α-MSH was not detected in +/+ mice.

Taken together, the results from **Paper II**, shows increased amounts of apoptotic cells and signs of degeneration of NPY fibers in the hypothalamus of the *anx/anx* mice.

4.4 DEFECTS IN THE OXPHOS SYSTEM IN THE *ANX/ANX* MOUSE (PAPER III & V)

4.4.1 First signs of OXPHOS dysfunction in *anx/anx* mice

In **Paper III**, a microarray analysis of the Arc from *anx/anx* and +/+ mice was performed. This analysis revealed 132 up regulated and 73 down regulated genes in *anx/anx* mice compared to +/+ mice, with ≥ 1.4-fold average increase or decrease. These genes with an altered expression level were included in an IPA analysis, to identify the top canonical pathways most likely to be involved in the phenotype of the *anx/anx* mouse. This resulted in several pathways centered around the OXPHOS system and mitochondrial dysfunction, as well as several genes related to mitochondrial function and oxidative stress (e.g. *Sod1*, *Prdx1*, *Bcl211*, and *Cox5B*). This was the first indication that the *anx* mutation could be involved in the mitochondrial OXPHOS system.

4.4.2 The *anx*-mutation and gene

In **Paper III**, we further investigated the *anx*-gene and searched for the *anx*-mutation, its biological function in, and relation to the phenotype of the *anx/anx* mouse. The *anx* gene has previously been localized to a 500-kb interval on mouse chromosome 2 [31]. Approximately 40 genes are found in the *anx*-interval. The microarray analysis gave us an overall insight in the different pathways affected in the *anx/anx* mice. However, it also directed our interest to the *Ndufaf1* gene that was among the down regulated genes found in the microarray. The *Ndufaf1* gene is located within the *anx*-interval and was in fact one out of two of the 40 genes in the interval that were affected in the array data. Based on that and on its biological function, the *Ndufaf1* gene became our number one *anx*-candidate gene. The downregulation of the *Ndufaf1* gene seen in the microarray analysis was verified by Taqman real time expression analysis. This showed a downregulation in the *Ndufaf1* gene in total brain, pancreas, liver and lung at mRNA level. However, no significant differences were found in kidney and heart, likely due to high variance in the sample set. Using a Western blot the downregulation of the *Ndufaf1* gene was also seen at protein level in total brain.

In the search for the *anx*-mutation we sequenced the *Ndufaf1* gene; however, no unique sequence alterations were found in coding sequences, neither in genomic DNA (exon) nor in cDNA. One explanation for this could be that the mutation could be located in a regulatory sequence such as a promotor region. This view is supported by the different expressions in various tissues.

When no unique sequence alterations were found, we continued by performing an allele-specific analysis. This was done to make sure that the downregulation of *Ndufaf1* was caused by the *anx*-mutation and was not due to a secondary effect, such as the starvation of the *anx/anx* mice. By the use of heterozygous (*anx/+)* mice, that carry one *anx*-allele and one wt-allele, the experiment could be carried out based on the fact that the *anx/+* mice do not show decreased body weight or aberrant neurological phenotype. By comparison of genomic DNA and cDNA levels of the *anx*-allele and the wt-allele a ratio was given. A ratio (cDNA versus genomic DNA) of 1 indicates no differences in allelic expression, and a ratio above one indicates a higher expression of the wt-allele. The results from this experiment showed, in all four cases studied, a ratio above one, suggesting that the downregulation of *Ndufaf1* expression levels in *anx/anx* mice is associated with the *anx*-allele of the *Ndufaf1* gene and is not due to a secondary effect of the phenotype.

4.4.3 Assembly and activity of hypothalamic OXPHOS CI in *anx/anx* mouse

The next step was to investigate potential effects of the downregulated *Ndufaf1* gene, i.e. the effect of the *anx*-mutation. The *Ndufaf1* gene encodes a mitochondrial CI assembly factor in the OXPHOS system [201]. CI is the largest of the five OXPHOS complexes, consisting of 45 different proteins. The role of *Ndufaf1* is to, together with other CI assembly factors (Tab. 1), build up a properly assembled mitochondrial CI. If this fails, the functions of CI are affected, i.e. the oxidation of NADH and the transport of electrons, which in the end could result in leakage of electrons, increased production of ROS and reduced production of ATP.

With the function of *Ndufaf1* in mind, as well as the microarray results suggesting a hypothalamic mitochondrial dysfunction in the *anx/anx* mouse, the levels of fully and partly assembled CI in *anx/anx* and +/+ mice were analyzed using BN PAGE. This revealed lower levels of fully assembled CI, as well as the presence of sub-complexes of CI, in the *anx/anx* hypothalamus.

Further, we analyzed the efficiency of the mitochondrial respiration in the hypothalamus of the *anx/anx* and +/+ mice using high-resolution respirometry. This analysis showed that the coupled respiration from hypothalamic CI is approximately 30% lower in *anx/anx* mice. The mitochondrial respiration via CII showed no significant difference comparing *anx/anx* and +/+ mice, indicating that the defects observed in the OXPHOS are specific for CI more and not a general defect of the respiratory chain. To exclude that the dysfunctions in the OXPHOS system in the *anx/anx* mice are due to a decrease in number of mitochondria, we performed a mitochondrial count. This showed that there is no difference in the number of mitochondria.

Thus, the aberrances in the OXPHOS system are not due to a decreased mitochondrial number in *anx/anx* mice.

The mitochondrial dysfunction seen in **Paper III** was confirmed in **Paper V** by evaluating the enzymatic activity of the different complexes in the respiratory chain in isolated mitochondria from hypothalamus of *anx/anx* and +/+ mice. A small but significant decrease in CI activity and in CI + CIII activity was seen in *anx/anx* compared to wt hypothalamus. No differences were seen in CII or in CII + CIII or in CIV activity. By using the mitochondrial marker citrate synthase (CS), the levels of mitochondria between *anx/anx* and +/+ mice were compared. No difference in CS activity was seen, indicating a similar level of mitochondria in the two groups. This confirmed that the mitochondrial CI dysfunction in the *anx/anx* mouse cannot be explained by a difference in mitochondrial content.

The results from **Paper III** and **Paper V** show a reduced efficiency of hypothalamic OXPHOS CI assembly and activity in the *anx/anx* mouse.

4.4.4 Oxidative stress in the *anx/anx* hypothalamus

The Affymetrix microarray study performed in **Paper III** revealed that several genes involved in oxidative stress are up regulated in the Arc in *anx/anx* mice, e.g. SOD1 and peroxiredoxin 1. Markers for ROS and oxidative stress were also analyzed by histochemical methods. We used dihydroetidium injections, which create a red fluorescent staining in the tissue when reacting with endogenous ROS. We could see increased levels of ROS in the *anx/anx* hypothalamus. Furthermore, we detected increased levels of SOD2, a mitochondrial scavenger of ROS. This shows an increased oxidative stress in *anx/anx* hypothalamus, possibly as an effect of the decreased hypothalamic levels of fully assembled CI.

The results from **Paper III** suggest that the phenotypes seen in the *anx/anx* mice, e.g. the anorexia and the hypothalamic neurodegeneration of appetite-regulating neurons, are associated with mitochondrial CI dysfunction that is related to an increase of ROS in the *anx/anx* hypothalamus.

4.5 MITOCHONDRIAL DYSFUNCTION AND INFLAMMATION IN ENDOCRINE PANCREAS (PAPER IV)

The hypothalamic inflammation reported in **Paper I** and **II**, and the hypothalamic mitochondrial dysfunction demonstrated in **Paper III**, led us to investigate whether the inflammation and OXPHOS dysfunction is also shown in endocrine pancreas in the *anx/anx* mouse.

4.5.1 Signs of mitochondrial dysfunction in *anx/anx* pancreatic islets

In **Paper IV** we detected a strong downregulation of the *Ndufaf1* gene in isolated islets from *anx/anx* mice. We therefore continued by studying the mitochondrial function in *anx/anx* islets. Using in-gel activity assay, we could detect a reduced mitochondrial CI activity in isolated *anx/anx* islets. To further analyze the mitochondrial function we measured the MMP when shifting from 3 to 11 mM glucose. MMP is related to the cells' capacity to generate ATP by oxidative phosphorylation, and is therefore an important indicator of cells' health or injury. We detected a 50% decreased MMP 3G/11G in *anx/anx* compared to wt isolated islets. The results from **Paper IV** suggest a mitochondrial dysfunction of *anx/anx* islets.

4.5.2 Signs of inflammation in *anx/anx* pancreatic islets

In **Paper IV**, we also investigated expression of inflammatory markers in *anx/anx* pancreatic islets and serum levels of FFAs. We studied inflammation with IHC analysis of intact pancreas and in isolated islets cultured overnight using antisera for Iba1 and F4/80, both markers for the macrophage lineage. We could detect a significantly increased macrophage infiltration in intact pancreas from *anx/anx* mice using double-labeling for Iba1 and insulin, indicating inflammation. However, in isolated islets cultured overnight no increased macrophage infiltration was seen. To establish the specificity of the experiment, we also carried out a double-labeling using Iba1 and F4/80 antiserum. This resulted in an almost complete overlap of the two markers. We continued by investigating the levels of free fatty acids in serum, and this revealed a 50% increase in free fatty acids in nonfasted *anx/anx* serum.

We then concluded that the difference in results, i.e. the inflammation seen in intact pancreas and the absence of inflammatory markers detected in isolated islets, could be due to a higher concentration of free fatty acids in in *anx/anx* serum, since the isolated islets are cultured in the absence of free fatty acids.

4.6 DISTURBED GLUCOSE HOMEOSTASIS AND INSULIN SECRETION IN THE *ANX/ANX* MOUSE (PAPER IV)

In **Paper IV**, we studied whole body glucose homeostasis and pancreatic islet function in the *anx/anx* mouse, monitoring several features such as insulin secretion and changes in free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$).

4.6.1 In vivo glucose tolerance test

In **Paper IV**, we studied whole body glucose homeostasis in *anx/anx* mice. We performed an ip glucose tolerance test (ipGTT) with 5-h-fasted or nonfasted *anx/anx* and wt mice. Thus, we evaluated the blood glucose levels and the serum insulin levels immediately before ip

administration of glucose (0 min) and after 30, 60 and 90 minutes. The blood glucose concentration in fasted *anx/anx* mice was significantly lower before ipGTT but markedly elevated after 30 minutes. This was associated with significantly decreased insulin serum levels. The nonfasted *anx/anx* mice showed lower blood glucose levels before ipGTT compared to wt mice; however, as in the fasted mice, markedly elevated blood glucose levels were seen after ip injection of glucose. We could conclude that *anx/anx* mice show a marked glucose intolerance associated with reduced insulin release.

We further investigated changes in β-cell mass, pancreatic hormone levels including total pancreatic insulin content and β-cell proliferation, to explain the disturbed glucose homeostasis in the *anx/anx* mouse. To study β-cell proliferation, double-labeling of insulin and Ki-67, a marker for proliferation, was carried out. This revealed an equal number of Ki-67-ir β-cells in *anx/anx* and wt pancreatic islets, i.e. no difference in β-cell proliferation was seen. We continued by investigating changes in β-cell mass by IHC. The amount of β-cells in *anx/anx* and wt islets was measured as the percentage of islet area occupied by insulin immunoreactivity, and did not differ between groups. Also, no significant differences were seen for the percentage of islet area occupied by glucagon or somatostatin. We analyzed pancreatic hormone levels by ELISA. Total glucagon content and total insulin content in *anx/anx* and wt pancreas normalized to total pancreatic protein did not show any significant difference. The lack of changes in β-cell mass, total pancreatic insulin content and β-cell proliferation suggests that the glucose intolerance and disturbed pancreatic islet insulin secretion cannot be explained by changes in these parameters.

4.6.2 In vitro insulin secretion and impaired $[Ca^{2+}]_i$ from *anx/anx* and wt islets

What could then be the cause for the glucose intolerance and the reduced insulin secretion seen in vivo following an ipGTT? To further investigate the cause for this we performed experiments on isolated islets to examine the insulin content and insulin secretion in vitro.

When measuring insulin content in isolated and cultured islets from *anx/anx* and wt mice we detected that *anx/anx* islets have a significantly reduced insulin content compared to wt islets after normalizing for protein content.

We also evaluated dynamic and static insulin secretion in response to glucose or KCl stimulation in isolated islets. When studying the islets in the presence of 3 mM glucose, representing the basal value, insulin concentration was similar between *anx/anx* and wt. However, after addition of 11 mM glucose, islets from *anx/anx* mice showed significantly higher insulin release compared to wt islets. In addition, depolarizing the islets with 25 mM

KCl also resulted in an increased insulin secretion from *anx/anx* compared to wt islets. Both dynamic and static insulin secretion showed the same results. The conclusion was that, even though isolated and cultured islets from *anx/anx* mice show lower insulin content, the insulin secretion from these islets, when stimulated with high glucose or KCl levels, is significantly increased compared to wt. This indicates that the reduced insulin release in response to the ipGTT in the *anx/anx* mice is not a cause of a decreased capacity to secrete insulin.

One important factor for a well-functioning insulin secretion is an adequate increase in free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$). We therefore continued by performing measurement of changes in $[Ca^{2+}]_i$, when islets from *anx/anx* and wt mice were stimulated with high glucose concentration, i.e. 11 mM or 25 mM KCl. Several differences showing an impaired $[Ca^{2+}]_i$ handling in *anx/anx* islets were observed.

When glucose concentration rises there are, during normal circumstances, an uptake of Ca^{2+} in the endoplasmic reticulum. In our experiment this can be observed as a first initial decrease in $[Ca^{2+}]_i$ right after increasing the glucose concentration from 3 to 11 mM. The first defect observed in *anx/anx* islets was a complete suppression of this decrease, whereas the wt islets showed a normal first initial decrease in $[Ca^{2+}]_i$ in all islets analyzed. After stimulating the islets with a high glucose concentration, the resulting increase in $[Ca^{2+}]_i$ was significantly lower in *anx/anx* compared to wt islets. Furthermore, when changing the glucose concentration from 11 mM to 3 mM, we detected a slower return of $[Ca^{2+}]_i$ to its basal levels in *anx/anx* versus wt islets. No differences were seen in $[Ca^{2+}]_i$ if stimulating the *anx/anx* islets with KCl, whereas KCl stimulation increased insulin release.

In **Paper IV**, we find that, following an ip injection of glucose, the *anx/anx* mouse shows glucose intolerance and reduced insulin release. However, insulin release from isolated *anx/anx* islets is increased after stimulation with glucose or KCl. The reduced insulin release following an glucose ip injection in vivo was not paralleled by the similar results in vitro, since isolated islets stimulated with high glucose or KCl showed increased insulin release. Further, we detected elevated levels of circulating FFAs in *anx/anx* serum and inflammation in intact pancreas but not in isolated islets cultured in the absence of FFAs. The conclusion of **Paper IV** is that the special characteristics of the *anx/anx* endocrine pancreas are dependent on the increased serum FFA levels and on the accompanying inflammation which inhibits the insulin secretion in vivo.

4.7 REDUCED HYPOTHALAMIC METABOLISM IN THE *ANX/ANX* MOUSE (PAPER V)

In **Paper III** we showed that mitochondrial CI in the OXPHOS system is dysfunctioning in the *anx/anx* mouse. This CI dysfunction is related to the aberrant expression of hypothalamic neuropeptides and transmitters regulating food intake. The *anx/anx* mouse shows an abnormal response to negative energy balance. In **Paper V** we further evaluated the reason for this abnormal response by investigating whether the *anx/anx* mouse shows a lower hypothalamic activity as a consequence of the dysfunctional CI. The hypothalamic activity, i.e. neuronal firing and transmitter release, is dependent of a well-functioning glucose utilization and energy metabolism. We therefore used these two processes to study neuronal activity.

In **Paper V**, hypothalamic activity of several metabolites was studied using both enzymatic analyzes and HPLC. PCr, Cr, ATP, lactate and G6P were analyzed by enzymatic analyzes, and IMP, ADP, ATP and AMP by HPLC. All metabolites were analyzed in the basal state and after one minute of ischemia in *anx/anx* and wt hypothalamus. These values were also used to calculate the anaerobic ATP turnover to assess hypothalamic metabolism under a stress condition, in this case ischemia. Hypothalamic protein levels of AMPK and glucose transporters were also measured, and [^{3}H]-2-deoxyglucose injections were made to evaluate hypothalamic glucose uptake.

The results regarding metabolites showed that both in the basal state and after ischemia PCr was significantly higher and lactate was significantly lower in *anx/anx* compared to wt hypothalamus. In the basal state, we saw no difference in ATP levels; however, after one minute of ischemia ATP levels were significantly higher in *anx/anx* compared to wt hypothalamus. When comparing the basal state to the ischemic state, the one minute of ischemia resulted in depletion of ATP and PCr, and accumulation of lactate in both *anx/anx* and wt hypothalamus.

To investigate the hypothalamic metabolism under a stress condition, i.e., in the ischemic hypothalamus, the anaerobic ATP turnover was calculated using the mean difference between ischemic and basal metabolite values assessed by HPLC, using the following formula: $-2\Delta ATP - \Delta PCr + (1.5\Delta$ lactate). Under this condition, glycolysis (lactate accumulation) was reduced by almost 40%, whereas anaerobic ATP turnover was ~10% lower in the *anx/anx* group.

During ischemia the AMP and IMP levels were decreased and the ATP levels were increased in the *anx/anx* hypothalamus. In addition, the ATP/AMP ratio was significantly increased in

anx/anx compared to wt hypothalamus. AMP and IMP are breakdown products of ATP, where IMP is a particularly sensitive indicator of net ATP degradation.

Further investigations on protein expression of AMPK, AMPK-P and GLUTs revealed significantly elevated protein levels of AMPK-T and GLUT4 in *anx/anx* compared to wt hypothalamus. Further, the ratio of AMPK-P/AMPK-T was decreased in *anx/anx* hypothalamus indicating a lower degree of AMPK activation

The rate of glucose uptake in the hypothalamus as well as in other brain areas, i.e. thalamus and motor cortex, as well as in the liver, was measured by injections with [^{3}H]-2-deoxy-glucose in *anx/anx* and wt mouse. Prior to the analysis, the mice were fasted for 5-6 hours. Significantly lower glucose uptake was observed in the *anx/anx* hypothalamus and in all other brain areas studied, as well as in the liver.

The results from **Paper V**, i.e. the reduced hypothalamic glucose uptake, the decreased activation state of AMPK, the reduced levels of AMP and IMP (breakdown products of ATP), and the increased levels of ATP and decreased levels of lactate after ischemia, all indicate a reduced hypothalamic metabolism, i.e. hypometabolism, in the *anx/anx* mouse both during basal and stress conditions.

5 CONCLUSIONS & HYPOTHESES

This thesis, based on the anorectic *anx/anx* mouse model, has been focused on advancing our understanding of the mechanisms leading to the anorectic phenotype of this mouse. Its unique characteristics, i.e. the starvation and the premature death, have previously been linked to aberrances and degeneration of discrete hypothalamic neuron populations expressing certain neuropeptides and transmitters of importance for the control of appetite. The studies included in this thesis provide the first evidence that the underlying cause for the anorectic phenotype in the *anx/anx* mouse is related to a mutation in one of the five complexes (CI-V) of the mitochondrial OXPHOS. More specific, we provide evidence for a downregulation of the gene for the CI assembly factor *Ndufaf1*, resulting in lower levels of CI, accumulation of sub-complexes, decreased CI activity and respiration and increased oxidative stress in the *anx/anx* mouse. We propose that this CI deficiency in the *anx/anx* hypothalamus may in fact represent a major contribution to the starvation of this mouse. We present evidence for this link between anorexia, CI dysfunction and increased levels of ROS in **Paper III**. A recent study further supports this link as it shows CI dysfunction and oxidative stress in leukocytes from patients with AN [197]. CI is the main producer of ROS even under normal conditions [170]. Results in **Paper III** indicate that a reduced capacity of CI can result in increased leakage of electrons from CI, producing even more ROS leading to oxidative stress. These results are interesting also because it is known that ROS has an additional role as signaling molecule, with direct effects on hypothalamic neurons and thereby regulating food intake; in fact, increased levels of ROS have been shown to inhibit food intake [167-169]. We therefore speculate that ROS at an early stage inhibits food intake in the *anx/anx* mouse. However, in the long run the increased levels of ROS can induce oxidative stress and inactivation and degeneration of certain Arc neurons in these mice [296, 297].

We evaluated both the activation state (**Paper V**) as well as the possible degeneration of the Arc neurons (**Paper I, II**). Indeed, we detected several signs of a neurodegenerative process in the hypothalamus of the *anx/anx* mice. Even if the *anx/anx* mouse shows a normal development of the AgRP/NPY system until P12, the normal increase in fiber density will then start to decrease; and at P21 the immunoreactivity for AgRP is distinctly decreased in nerve terminals, and dramatically increased in cell bodies. This process overlaps both in time and space with the activation of microglia, and with increases of MHC class I mRNA and protein both in microglia and Arc neurons expressing Y1 or AgRP. We could also see that the POMC/CART neurons are reduced in *anx/anx* mice at P21. In addition, we detected increased number of apoptotic cells in the hypothalamus of *anx/anx* mice by using TUNEL-labeling, as well as

demonstrating co-labeling of NPY and actCasp6, a marker for axonal degeneration, in the Arc and several other hypothalamic brain areas. Taken together, these results strongly suggest a degenerative process in the *anx/anx* hypothalamus.

Furthermore, our results in **Paper II** suggest that the activation state of Arc neurons is changed in the *anx/anx* mice. Several studies have shown that neurons are more likely to express MHC class I, when they are in a silent or in an inactive state, compared to active neurons [290, 298]. We detected that the *anx/anx* Arc NPY/AgRP and POMC/CART neurons expressing MHC class I have very low levels of Fos B, a marker for cellular activity, indicating a silenced or inactive state. This could be a consequence of the CI deficiency, and thus low ATP levels, with the aim to prevent cell death of these neurons. Another role for MHC class I molecules is to preserve synapses and to induce regeneration after injury [292]. Thus, the upregulated MHC class I molecules in the Arc neurons could protect these neurons from synaptic elimination. Indeed, hypothalamic activity is dependent on a well-functioning energy metabolism.

In **Paper V**, we further evaluated the activation state of the *anx/anx* hypothalamus by investigating the metabolism in this brain region. The *anx/anx* hypothalamus showed a lower rate of glucose uptake, decreased lactate content and increased PCr content in the basal state. This reflects a low metabolic stress, hypometabolism and a lower neuronal activity. Similar changes have been reported in AD patients [299-301].

Impaired oxidative phosphorylation has previously been linked to β-cell dysfunction and the development of diabetes [132, 257-261]. Also, in **Paper III**, we detected a downregulation of the *Ndufaf1* gene not only in brain but also in pancreas. We therefore continued to study the function of the endocrine pancreas in the *anx/anx* mouse. The results from **Paper IV** gave us new and valuable insights in the association between anorexia, mitochondrial dysfunction, inflammation, glucose intolerance and β-cell dysfunction. Following an ipGTT the *anx/anx* mice showed glucose intolerance and reduced insulin secretion. We also detected elevated levels of FFA in *anx/anx* serum and increased macrophage infiltration and inflammation in *anx/anx* islets from intact pancreas. However, when isolated *anx/anx* islets were cultured in the absence of FFAs, insulin secretion was no longer reduced. In line with this, no macrophage infiltration was seen in isolated and cultured *anx/anx* islets. Macrophages have been shown to release proinflammatory cytokines, that are known to inhibit insulin secretion [302, 303]. Thus, the increased serum levels of FFAs and the macrophage infiltration and inflammation in islets from intact pancreas in the *anx/anx* mouse may be responsible for the decreased insulin secretion and inflammation seen in vivo. Further support for this hypothesis is seen in several studies that have related chronically increased levels of FFAs to both inflammation and

metabolic disorders. Increased levels of FFAs have been observed in both obesity, AN and in diabetes [258, 259, 304]. In obesity, the high levels of FFAs are due to the increased fat deposits and in AN it is due to an increased breakdown of the fat deposits [304-306]. Furthermore, elevated levels of FFAs can also inhibit glucose-induced insulin secretion and result in pancreatic inflammation and β-cell dysfunction [307, 308].

In **Paper IV** we also report on changes in $[Ca^{2+}]_i$ in response to high glucose concentrations. After stimulating the islets with high glucose concentration, the resulting increase in $[Ca^{2+}]_i$ was significantly lower in *anx/anx* compared to wt islets. No differences were seen when $[Ca^{2+}]_i$ was stimulated with KCl. Why do we see an alteration in $[Ca^{2+}]_i$ in response to glucose and not in response to KCl? A likely explanation is that KCl acts directly on the voltage-gated calcium channels, causing Ca^{2+} influx and insulin secretion. In contrast, glucose causes insulin secretion via uptake by GLUT2, increased ATP production, closure of the ATP-sensitive potassium channel, membrane depolarization and then opening of voltage-gated Ca^{2+} channels. This indicates that the cause for the decreased $[Ca^{2+}]_i$ in response to glucose must lie upstream of the voltage-gated calcium channels. We suggest that the decreased CI activity and impaired change in MMP found in the *anx/anx* islets contribute to the impaired $[Ca^{2+}]_i$ when changing the glucose concentration from low to high. Thus, **Paper IV** provides a link between anorexia, CI dysfunction and β-cell dysfunction. That goes in line with the fact that diabetic patients show an increased risk of developing an eating disorders, and that OXPHOS dysfunctions are linked to the development of diabetes [18, 19, 304-306, 309].

The papers in this thesis show that the phenotypes of the *anx/anx* mouse so far are related mostly to the brain and the pancreas. In the brain, the aberrances and the neurodegeneration are specifically seen in the hypothalamus and likely not related to the whole brain, even though the downregulation of the *Ndufaf1* mRNA and protein is seen in total brain. How come that the mutation in the *Ndufaf1* gene and the subsequent CI dysfunction and oxidative stress only affect specific tissues and brain regions? An explanation could be that some factors make specific tissues and brain regions extra sensitive to CI deficiency and oxidative stress. One such factor of interest is a specific type of ATP-sensitive potassium channels (K^{+}_{-ATP}) [310-312]. These channels consist of two types of subunits, the pore-forming potassium channel (Kir6-) and the regulatory sulphonylurea receptor subunit (SUR) [313, 314]. This type of K^{+}_{-ATP} channels have been shown to be extra sensitive to CI inhibition [314] and become activated by mitochondrial CI dysfunction, leading to reduced neuronal activity, i.e. lower neuronal firing. They are expressed by some hypothalamic neurons such as the POMC/CART and AgRP/NPY neurons, and by the dopaminergic neurons in the substantia nigra as well as by the pancreatic β-cells

[310-312, 315]. These channels are regulated by intracellular ATP so that high levels of intracellular ATP close the channel [316]. This also means that during times of ATP depletion, such as metabolic stress and ischemia, the K^+-ATP channels are open, resulting in reduced neuronal activity and neurotransmitter release, in order to save ATP [317]. This represents a neuroprotective role of the Kir6.2/SUR1 K^+-ATP channels [317]. However, during times of chronically reduced neuronal activity, the gene and protein expression important for neuronal survival will decrease, leading to neurodegeneration [318, 319]. Thus, short-term activation of the neuronal K^+-ATP channels can protect the neurons against metabolic stress, whereas a chronic activation of the K^+-ATP channels is dangerous to the cell. This goes in line with the findings in the *anx/anx* mouse, showing CI deficiency and reduced neuronal activity in hypothalamus: the CI deficiency could result in inactivation of the hypothalamic appetite regulating neurons expressing the Kir6.2/SUR1 K+-ATP channels, and in the longer perspective degeneration of these neurons.

In conclusion, the results from this thesis suggest that the anorexia and disturbed feeding behavior in the *anx/anx* mouse as well as the hypothalamic neurodegeneration of appetite controlling neurons in the Arc of the *anx/anx* mouse are related to a mitochondrial CI dysfunction, caused by a downregulation of the CI assembly factor *Ndufaf1* gene. Both neurodegeneration and poor feeding in infants are attributes commonly seen in humans with disorders caused by OXPHOS CI dysfunctions. Furthermore, the *anx/anx* mouse shows glucose intolerance, disturbed insulin secretion and pancreatic β-cell dysfunction that are related to mitochondrial dysfunction, increased FFA in serum and inflammation in islets. Both inflammation and impaired oxidative phosphorylation have previously been linked to β-cell dysfunction and the development of diabetes [132, 257-261]. Increased levels of circulation FFA, which are involved in the generation of inflammation [258, 259], are seen both in diabetic and obese, as well as in AN patient [304-306, 309]. Furthermore, a group with increased risk of developing AN is patients suffering from diabetes [18, 19, 23]. Several of the phenotypes observed in the *anx/anx* mouse are also seen in AN patient. These include, self-starvation, mitochondrial dysfunction and increased levels of FFA. Taken together, the results from this thesis work, when seen against the background of a spectrum of human disorders, show the value of the *anx/anx* mouse to gain a better understanding of conditions involving a dysregulated appetite. Perhaps, in the long run, they may even lead to new therapeutic methods of such conditions.

6 FUTURE PERSPECTIVES

As discussed earlier in this thesis, the best treatment methods for AN still need to be found. As for today, the most important factor for full recovery is early treatment and nutrient restoration. This thesis provides a link between anorexia and CI dysfunction. When it comes to CI deficiencies, there is currently no cure and the therapeutic strategies can relive symptoms but are unable to cure or even delay the progress of the disease.

There are several aspects of the anorectic biology of the *anx/anx* mouse that need to be studied. The role of metabolic alterations relating to fatty acids should be investigated further as it seems to play a critical role in the pancreas. Transgenic models may be used to rescue the phenotype, perhaps involving overexpression of specific components of CI or overexpression of the *Ndufaf1* gene.

The correlation between anorexia, mitochondrial deficiency and CI dysfunction found in the studies in this thesis helped us come one step closer to a better understanding of mechanisms underlying the development of anorectic behavior. Thereby better therapeutic treatment methods may emerge that could lead to improvement and perhaps even recovery from various kinds of eating disorders. Is it possible to define a subgroup of patients with anorexia with a clearcut loss of hypothalamic neurons? Would it be possible to intervene more aggressively in this group to avoid cell death? There are already survival promoting factors used in clinical trials in other neurological conditions involving excessive cell death. Could some of these be used or could it be that prevention is more critical with intensive nutritional intervention early in the disease? This would require development of biomarkers that can be applied as a screening tool very early or even before disease onset in at risk individuals.

A recent study done by Victor et. al. [197] showed CI dysfunction and oxidative stress in leukocytes from AN patient. To control that this CI dysfunction and oxidative stress is not due to a secondary effect caused by the starvation but rather a part of the underlying biology causing this behavior, one could further investigate these components in healthy individuals previously struggling with AN.

A future step would be in a curable manner, taking a mitochondrial approach to find new treatment methods for different kinds of eating disorders, and as a first step to cure the *anx/anx* mouse. Several approaches could be taken, for example, to treat the *anx/anx* mice with anti-inflammatory and anti-oxidative compounds. This could also be taken to the clinic, as a complement to the already existing treatment, one could enhance the AN treatment with anti-inflammatory and anti-oxidative compounds. One could also try to improve the activity of CI

by treatment with riboflavin, as seen in patients with mutation in the CI assembly factor. This treatment resulted in increased CI activity and improved the clinical outcome of these patients [229]. This would require well controlled randomized controlled trials, most likely combined with a biomarker approach to a priori identify patients with an increase in oxidative stress or less active C1 in the hypothalamus. Whereas imaging techniques may be one avenue towards identifying this potential subgroup of patients, it may in fact detect individuals only after damage to neurons in the hypothalamus has occurred. Imaging research may therefore have to be combined with biochemical or molecular strategies to identify trait markers. This approach would be a way of introducing individualized medicine into the field of eating disorders.

7 ACKNOWLEDGEMENT

First of all, I would like to give my greatest thanks to my supervisors. Ida Nilsson, your support as a supervisor, colleague and friend has been priceless and this work would not have been made without you. Your knowledge of the field has been absolutely critical.

Martin Schalling, thank you for giving me the possibility to do research in your lab, for your encouragement and understanding. Thank you for establishing the *anx/anx* model and being the founder to the *anx*-project at Karolinska Institutet, and for asking the right scientific questions that took the *anx*-project to its current position. Tomas Hökfelt, thank you for your valuable input and help in writing and being ready to help in any way you can. Roger Adan, thank you for being my supervisor and for your inspirational work in the anorexia field.

Jeanette Johansen, thank you for always taking the time to answer all of my questions and discussing results. Abram Katz, Pablo Garcia Roves and Sergei Saitzev, I always enjoyed coming to your labs. Thank you for sharing your knowledge in your specialized field of research.

I would also like to give a special thanks to all my co-authors for great collaboration, Sergueï O. Fetissov, Sebastian Thams, Anita Bergstrand, Staffan Cullheim, Aamir R. Zuberi, Mohsen Karimi, Leah Rae Donahue, Derry C. Roopenian, Jan Mulder, Mathias Uhlen, Tomas J. Ekström, Muriel T. Davisson, Lars Selander, Giulia Marconi, Per-Olof Berggren, Ulrika Bergström, Marie Svedberg, Jenny Häggkvist, Rolf Wibom.

All my colleagues and friends at the Neurogenetic lab. You have made the work easy and fun. Being at this lab together with all of you, has been a joyful time that I will never forget.

My family, you are priceless to me, thank you for all your help, especially during this last part of my PhD time, I could not have done it without you. I would also like to thank my precious daughter Linnea for drawing the nice front picture on this thesis.

8 REFERENCES

1. Reaven, P., et al., *Cardiovascular disease insulin risk in Mexican-American and Anglo-American children and mothers.* Pediatrics, 1998. **101**(4): p. E12.

2. McTiernan, A., *Obesity and cancer: the risks, science, and potential management strategies.* Oncology (Williston Park), 2005. **19**(7): p. 871-81; discussion 881-2, 885-6.

3. Bulik, C.M., et al., *Prevalence, heritability, and prospective risk factors for anorexia nervosa.* Arch Gen Psychiatry, 2006. **63**(3): p. 305-12.

4. Kaye, W., *Neurobiology of anorexia and bulimia nervosa.* Physiol Behav, 2008. **94**(1): p. 121-35.

5. Tan, B.H. and K.C. Fearon, *Cachexia: prevalence and impact in medicine.* Curr Opin Clin Nutr Metab Care, 2008. **11**(4): p. 400-7.

6. Inui, A., *Cancer anorexia-cachexia syndrome: current issues in research and management.* CA Cancer J Clin, 2002. **52**(2): p. 72-91.

7. Nelson, K.A., *The cancer anorexia-cachexia syndrome.* Semin Oncol, 2000. **27**(1): p. 64-8.

8. Ramos, E.J., et al., *Cancer anorexia-cachexia syndrome: cytokines and neuropeptides.* Curr Opin Clin Nutr Metab Care, 2004. **7**(4): p. 427-34.

9. Shields, B., I. Wacogne, and C.M. Wright, *Weight faltering and failure to thrive in infancy and early childhood.* BMJ, 2012. **345**: p. e5931.

10. Olsen, E.M., et al., *Risk factors for weight faltering in infancy according to age at onset.* Paediatr Perinat Epidemiol, 2010. **24**(4): p. 370-82.

11. Kasese-Hara, M., C. Wright, and R. Drewett, *Energy compensation in young children who fail to thrive.* J Child Psychol Psychiatry, 2002. **43**(4): p. 449-56.

12. Shaoul, R., et al., *Leptin and cytokines levels in children with failure to thrive.* J Pediatr Gastroenterol Nutr, 2003. **37**(4): p. 487-91.

13. Stice, E., *Risk and maintenance factors for eating pathology: a meta-analytic review.* Psychol Bull, 2002. **128**(5): p. 825-48.

14. McNutt, S.W., et al., *A longitudinal study of the dietary practices of black and white girls 9 and 10 years old at enrollment: the NHLBI Growth and Health Study.* J Adolesc Health, 1997. **20**(1): p. 27-37.

15. Gustafson-Larson, A.M. and R.D. Terry, *Weight-related behaviors and concerns of fourth-grade children.* J Am Diet Assoc, 1992. **92**(7): p. 818-22.

16. Boutelle, K., et al., *Weight control behaviors among obese, overweight, and nonoverweight adolescents.* J Pediatr Psychol, 2002. **27**(6): p. 531-40.

17. Shisslak, C.M., M. Crago, and L.S. Estes, *The spectrum of eating disturbances.* Int J Eat Disord, 1995. **18**(3): p. 209-19.

18. Hudson, J.I., et al., *Prevalence of anorexia nervosa and bulimia among young diabetic women.* J Clin Psychiatry, 1985. **46**(3): p. 88-9.

19. Rodin, G.M., et al., *Eating disorders in female adolescents with insulin dependent diabetes mellitus.* Int J Psychiatry Med, 1986. **16**(1): p. 49-57.

20. Davidson, J., *Diabulimia: how eating disorders can affect adolescents with diabetes.* Nurs Stand, 2014. **29**(2): p. 44-9.

21. Colton, P.A., et al., *Eating Disorders in Girls and Women With Type 1 Diabetes: A Longitudinal Study of Prevalence, Onset, Remission, and Recurrence.* Diabetes Care, 2015. **38**(7): p. 1212-7.

22. Casiero, D. and W.H. Frishman, *Cardiovascular complications of eating disorders.* Cardiol Rev, 2006. **14**(5): p. 227-31.

23. Mangweth, B., et al., *Family study of the aggregation of eating disorders and mood disorders.* Psychol Med, 2003. **33**(7): p. 1319-23.

24. McElroy, S.L., R. Kotwal, and P.E. Keck, Jr., *Comorbidity of eating disorders with bipolar disorder and treatment implications.* Bipolar Disord, 2006. **8**(6): p. 686-95.

25. Swanson, S.A., et al., *Prevalence and correlates of eating disorders in adolescents. Results from the national comorbidity survey replication adolescent supplement.* Arch Gen Psychiatry, 2011. **68**(7): p. 714-23.

26. Papadopoulos, F.C., et al., *Excess mortality, causes of death and prognostic factors in anorexia nervosa.* Br J Psychiatry, 2009. **194**(1): p. 10-7.

27. Sullivan, P.F., *Mortality in anorexia nervosa.* Am J Psychiatry, 1995. **152**(7): p. 1073-4.

28. Hoek, H.W., *Incidence, prevalence and mortality of anorexia nervosa and other eating disorders.* Curr Opin Psychiatry, 2006. **19**(4): p. 389-94.

29. Arcelus, J., et al., *Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies.* Arch Gen Psychiatry, 2011. **68**(7): p. 724-31.

30. Hinney, A., et al., *Evidence for three genetic loci involved in both anorexia nervosa risk and variation of body mass index.* Mol Psychiatry, 2017. **22**(2): p. 321-322.

31. Maltais, L.J., P.W. Lane, and W.G. Beamer, *Anorexia, a recessive mutation causing starvation in preweanling mice.* J Hered, 1984. **75**(6): p. 468-72.

32. Chronwall, B.M., *Anatomy and physiology of the neuroendocrine arcuate nucleus.* Peptides, 1985. **6 Suppl 2**: p. 1-11.

33. Everitt, B.J., et al., *The hypothalamic arcuate nucleus-median eminence complex: immunohistochemistry of transmitters, peptides and DARPP-32 with special reference to coexistence in dopamine neurons.* Brain Res, 1986. **396**(2): p. 97-155.

34. Meister, B., *Control of food intake via leptin receptors in the hypothalamus.* Vitam Horm, 2000. **59**: p. 265-304.

35. Sawchenko, P.E., *Toward a new neurobiology of energy balance, appetite, and obesity: the anatomists weigh in.* J Comp Neurol, 1998. **402**(4): p. 435-41.

36. Lawrence, C.B., A.V. Turnbull, and N.J. Rothwell, *Hypothalamic control of feeding.* Curr Opin Neurobiol, 1999. **9**(6): p. 778-83.

37. Meister, B., *Gene expression and chemical diversity in hypothalamic neurosecretory neurons.* Mol Neurobiol, 1993. **7**(2): p. 87-110.

38. Schwartz, M.W., et al., *Central nervous system control of food intake.* Nature, 2000. **404**(6778): p. 661-71.

39. Broberger, C. and T. Hokfelt, *Hypothalamic and vagal neuropeptide circuitries regulating food intake.* Physiol Behav, 2001. **74**(4-5): p. 669-82.

40. Cone, R.D., et al., *The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis.* Int J Obes Relat Metab Disord, 2001. **25 Suppl 5**: p. S63-7.

41. Schwartz, M.W., *Brain pathways controlling food intake and body weight.* Exp Biol Med (Maywood), 2001. **226**(11): p. 978-81.

42. Hillebrand, J.J., D. de Wied, and R.A. Adan, *Neuropeptides, food intake and body weight regulation: a hypothalamic focus.* Peptides, 2002. **23**(12): p. 2283-306.

43. Saper, C.B., T.C. Chou, and J.K. Elmquist, *The need to feed: homeostatic and hedonic control of eating.* Neuron, 2002. **36**(2): p. 199-211.

44. Druce, M. and S.R. Bloom, *Central regulators of food intake.* Curr Opin Clin Nutr Metab Care, 2003. **6**(4): p. 361-7.

45. van den Pol, A.N., *Weighing the role of hypothalamic feeding neurotransmitters.* Neuron, 2003. **40**(6): p. 1059-61.

46. Kalra, S.P. and P.S. Kalra, *NPY and cohorts in regulating appetite, obesity and metabolic syndrome: beneficial effects of gene therapy.* Neuropeptides, 2004. **38**(4): p. 201-11.

47. Broberger, C., *Brain regulation of food intake and appetite: molecules and networks.* J Intern Med, 2005. **258**(4): p. 301-27.

48. Leibowitz, S.F., *Regulation and effects of hypothalamic galanin: relation to dietary fat, alcohol ingestion, circulating lipids and energy homeostasis.* Neuropeptides, 2005. **39**(3): p. 327-32.

49. Hillebrand, J.J., M.J. Kas, and R.A. Adan, *To eat or not to eat; regulation by the melanocortin system.* Physiol Behav, 2006. **89**(1): p. 97-102.

50. Schneeberger, M., R. Gomis, and M. Claret, *Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance.* J Endocrinol, 2014. **220**(2): p. T25-46.

51. Cheng, X., et al., *Regulation of expression of neuropeptide Y Y1 and Y2 receptors in the arcuate nucleus of fasted rats.* Brain Res, 1998. **792**(1): p. 89-96.

52. Cone, R.D., *Anatomy and regulation of the central melanocortin system.* Nat Neurosci, 2005. **8**(5): p. 571-8.

53. Kristensen, P., et al., *Hypothalamic CART is a new anorectic peptide regulated by leptin.* Nature, 1998. **393**(6680): p. 72-6.

54. Vrang, N., et al., *Neurochemical characterization of hypothalamic cocaine-amphetamine-regulated transcript neurons.* J Neurosci, 1999. **19**(10): p. RC5.

55. Vrang, N., et al., *Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour.* Brain Res, 1999. **818**(2): p. 499-509.

56. Kim, M.S., et al., *The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin.* J Clin Invest, 2000. **105**(7): p. 1005-11.

57. Yasuda, T., et al., *Hypothalamic melanocortin system regulates sympathetic nerve activity in brown adipose tissue.* Exp Biol Med (Maywood), 2004. **229**(3): p. 235-9.

58. Allen, Y.S., et al., *Neuropeptide Y distribution in the rat brain.* Science, 1983. **221**(4613): p. 877-9.

59. Chronwall, B.M., et al., *The anatomy of neuropeptide-Y-containing neurons in rat brain.* Neuroscience, 1985. **15**(4): p. 1159-81.

60. de Quidt, M.E. and P.C. Emson, *Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system--I. Radioimmunoassay and chromatographic characterisation.* Neuroscience, 1986. **18**(3): p. 527-43.

61. Clark, J.T., et al., *Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats.* Endocrinology, 1984. **115**(1): p. 427-9.

62. Rossi, M., et al., *A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone in vivo.* Endocrinology, 1998. **139**(10): p. 4428-31.

63. Ollmann, M.M., et al., *Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein.* Science, 1997. **278**(5335): p. 135-8.

64. Broberger, C., et al., *Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems.* J Comp Neurol, 1998. **402**(4): p. 460-74.

65. Hahn, T.M., et al., *Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons.* Nat Neurosci, 1998. **1**(4): p. 271-2.

66. Krashes, M.J., et al., *Rapid, reversible activation of AgRP neurons drives feeding behavior in mice.* J Clin Invest, 2011. **121**(4): p. 1424-8.

67. Aponte, Y., D. Atasoy, and S.M. Sternson, *AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training.* Nat Neurosci, 2011. **14**(3): p. 351-5.

68. Marks, J.L., et al., *Localization of insulin receptor mRNA in rat brain by in situ hybridization.* Endocrinology, 1990. **127**(6): p. 3234-6.

69. Hakansson, M.L., A.L. Hulting, and B. Meister, *Expression of leptin receptor mRNA in the hypothalamic arcuate nucleus--relationship with NPY neurones.* Neuroreport, 1996. **7**(18): p. 3087-92.

70. Elmquist, J.K., et al., *Distributions of leptin receptor mRNA isoforms in the rat brain.* J Comp Neurol, 1998. **395**(4): p. 535-47.

71. Obici, S., et al., *Hypothalamic insulin signaling is required for inhibition of glucose production.* Nat Med, 2002. **8**(12): p. 1376-82.

72. Wren, A.M., et al., *Ghrelin enhances appetite and increases food intake in humans.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5992.

73. Wren, A.M., et al., *The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion.* Endocrinology, 2000. **141**(11): p. 4325-8.

74. Kirsz, K. and D.A. Zieba, *Ghrelin-mediated appetite regulation in the central nervous system.* Peptides, 2011. **32**(11): p. 2256-64.

75. Watson, S.J., J.D. Barchas, and C.H. Li, *beta-Lipotropin: localization of cells and axons in rat brain by immunocytochemistry.* Proc Natl Acad Sci U S A, 1977. **74**(11): p. 5155-8.

76. Bai, F.L., et al., *An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat.* Brain Res, 1985. **331**(1): p. 172-5.

77. Broberger, C., et al., *The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 15043-8.

78. Wynne, K., et al., *Appetite control.* J Endocrinol, 2005. **184**(2): p. 291-318.

79. Baldo, B.A., et al., *Overlapping distributions of orexin/hypocretin- and dopamine-beta-hydroxylase immunoreactive fibers in rat brain regions mediating arousal, motivation, and stress.* J Comp Neurol, 2003. **464**(2): p. 220-37.

80. Moga, M.M., R.P. Weis, and R.Y. Moore, *Efferent projections of the paraventricular thalamic nucleus in the rat.* J Comp Neurol, 1995. **359**(2): p. 221-38.

81. Larsen, P.J., A. Hay-Schmidt, and J.D. Mikkelsen, *Efferent connections from the lateral hypothalamic region and the lateral preoptic area to the hypothalamic paraventricular nucleus of the rat.* J Comp Neurol, 1994. **342**(2): p. 299-319.

82. Butler, A.A., et al., *Melanocortin-4 receptor is required for acute homeostatic responses to increased dietary fat.* Nat Neurosci, 2001. **4**(6): p. 605-11.

83. Schwartz, M.W. and G.J. Morton, *Obesity: keeping hunger at bay.* Nature, 2002. **418**(6898): p. 595-7.

84. Minokoshi, Y., et al., *AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus.* Nature, 2004. **428**(6982): p. 569-74.

85. Barsh, G.S. and M.W. Schwartz, *Genetic approaches to studying energy balance: perception and integration.* Nat Rev Genet, 2002. **3**(8): p. 589-600.

86. Hardie, D.G., *AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 774-85.

87. Lopez, M., et al., *Hypothalamic AMPK: a canonical regulator of whole-body energy balance.* Nat Rev Endocrinol, 2016. **12**(7): p. 421-32.

88. Schneeberger, M. and M. Claret, *Recent Insights into the Role of Hypothalamic AMPK Signaling Cascade upon Metabolic Control.* Front Neurosci, 2012. **6**: p. 185.

89. Minokoshi, Y., et al., *Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase.* Nature, 2002. **415**(6869): p. 339-43.

90. Andersson, U., et al., *AMP-activated protein kinase plays a role in the control of food intake.* J Biol Chem, 2004. **279**(13): p. 12005-8.

91. Lopez, M., et al., *Hypothalamic fatty acid metabolism mediates the orexigenic action of ghrelin.* Cell Metab, 2008. **7**(5): p. 389-99.

92. Gao, S., et al., *Differential effects of central ghrelin on fatty acid metabolism in hypothalamic ventral medial and arcuate nuclei.* Physiol Behav, 2013. **118**: p. 165-70.

93. Lim, C.T., et al., *Ghrelin and cannabinoids require the ghrelin receptor to affect cellular energy metabolism.* Mol Cell Endocrinol, 2013. **365**(2): p. 303-8.

94. Bennett, P.A., et al., *Hypothalamic growth hormone secretagogue-receptor (GHS-R) expression is regulated by growth hormone in the rat.* Endocrinology, 1997. **138**(11): p. 4552-7.

95. Hardie, D.G., et al., *AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge.* Biochem J, 1999. **338 (Pt 3)**: p. 717-22.

96. Hardie, D.G. and D. Carling, *The AMP-activated protein kinase--fuel gauge of the mammalian cell?* Eur J Biochem, 1997. **246**(2): p. 259-73.

97. Moore, F., J. Weekes, and D.G. Hardie, *Evidence that AMP triggers phosphorylation as well as direct allosteric activation of rat liver AMP-activated protein kinase. A sensitive mechanism to protect the cell against ATP depletion.* Eur J Biochem, 1991. **199**(3): p. 691-7.

98. Marsin, A.S., et al., *Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia.* Curr Biol, 2000. **10**(20): p. 1247-55.

99. Marsin, A.S., et al., *The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase.* J Biol Chem, 2002. **277**(34): p. 30778-83.

100. Berasi, S.P., et al., *Inhibition of gluconeogenesis through transcriptional activation of EGR1 and DUSP4 by AMP-activated kinase.* J Biol Chem, 2006. **281**(37): p. 27167-77.

101. Horman, S., et al., *Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis.* Curr Biol, 2002. **12**(16): p. 1419-23.

102. Clarke, P.R. and D.G. Hardie, *Regulation of HMG-CoA reductase: identification of the site phosphorylated by the AMP-activated protein kinase in vitro and in intact rat liver.* EMBO J, 1990. **9**(8): p. 2439-46.

103. Muoio, D.M., et al., *AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that sn-glycerol-3-phosphate acyltransferase is a novel target.* Biochem J, 1999. **338 (Pt 3)**: p. 783-91.

104. McCrimmon, R.J., et al., *Key role for AMP-activated protein kinase in the ventromedial hypothalamus in regulating counterregulatory hormone responses to acute hypoglycemia.* Diabetes, 2008. **57**(2): p. 444-50.

105. Kim, M.S., et al., *Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase.* Nat Med, 2004. **10**(7): p. 727-33.

106. Alquier, T., et al., *Role of hypothalamic adenosine 5'-monophosphate-activated protein kinase in the impaired counterregulatory response induced by repetitive neuroglucopenia.* Endocrinology, 2007. **148**(3): p. 1367-75.

107. McCrimmon, R.J., et al., *Potential role for AMP-activated protein kinase in hypoglycemia sensing in the ventromedial hypothalamus.* Diabetes, 2004. **53**(8): p. 1953-8.

108. Claret, M., et al., *AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons.* J Clin Invest, 2007. **117**(8): p. 2325-36.

109. Fujii, N., et al., *Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle.* Biochem Biophys Res Commun, 2000. **273**(3): p. 1150-5.

110. Davalos, D., et al., *ATP mediates rapid microglial response to local brain injury in vivo.* Nat Neurosci, 2005. **8**(6): p. 752-8.

111. Nimmerjahn, A., F. Kirchhoff, and F. Helmchen, *Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo.* Science, 2005. **308**(5726): p. 1314-8.

112. Aloisi, F., *Immune function of microglia.* Glia, 2001. **36**(2): p. 165-79.

113. Kreutzberg, G.W., *Microglia: a sensor for pathological events in the CNS.* Trends Neurosci, 1996. **19**(8): p. 312-8.

114. Graeber, M.B., W.J. Streit, and G.W. Kreutzberg, *Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells.* J Neurosci Res, 1988. **21**(1): p. 18-24.

115. Colton, C.A. and D.L. Gilbert, *Production of superoxide anions by a CNS macrophage, the microglia.* FEBS Lett, 1987. **223**(2): p. 284-8.

116. Hurley, S.D., et al., *Cytokine transcripts expressed by microglia in vitro are not expressed by ameboid microglia of the developing rat central nervous system.* Glia, 1999. **25**(3): p. 304-9.

117. Block, M.L. and J.S. Hong, *Chronic microglial activation and progressive dopaminergic neurotoxicity.* Biochem Soc Trans, 2007. **35**(Pt 5): p. 1127-32.

118. Zinkernagel, R.M. and P.C. Doherty, *Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis.* Nature, 1974. **251**(5475): p. 547-8.

119. Foster, J.A., et al., *Induced neuronal expression of class I major histocompatibility complex mRNA in acute and chronic inflammation models.* J Neuroimmunol, 2002. **131**(1-2): p. 83-91.

120. Linda, H., et al., *Expression of MHC class I and beta2-microglobulin in rat spinal motoneurons: regulatory influences by IFN-gamma and axotomy.* Exp Neurol, 1998. **150**(2): p. 282-95.

121. Schultzberg, M., et al., *Early major histocompatibility complex (MHC) class I antigen induction in hypothalamic supraoptic and paraventricular nuclei in trypanosome-infected rats.* J Neuroimmunol, 1989. **24**(1-2): p. 105-12.

122. Thams, S., A. Oliveira, and S. Cullheim, *MHC class I expression and synaptic plasticity after nerve lesion.* Brain Res Rev, 2008. **57**(1): p. 265-9.

123. Moffett, C.W. and C.M. Paden, *Microglia in the rat neurohypophysis increase expression of class I major histocompatibility antigens following central nervous system injury.* J Neuroimmunol, 1994. **50**(2): p. 139-51.

124. Neumann, H., *Control of glial immune function by neurons.* Glia, 2001. **36**(2): p. 191-9.

125. Odegaard, J.I. and A. Chawla, *The immune system as a sensor of the metabolic state.* Immunity, 2013. **38**(4): p. 644-54.

126. Odegaard, J.I. and A. Chawla, *Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis.* Science, 2013. **339**(6116): p. 172-7.

127. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-7.

128. Pimentel, G.D., K. Ganeshan, and J.B. Carvalheira, *Hypothalamic inflammation and the central nervous system control of energy homeostasis.* Mol Cell Endocrinol, 2014. **397**(1-2): p. 15-22.

129. Argente-Arizon, P., et al., *Role of non-neuronal cells in body weight and appetite control.* Front Endocrinol (Lausanne), 2015. **6**: p. 42.

130. Zhang, X., et al., *Hypothalamic IKKbeta/NF-kappaB and ER stress link overnutrition to energy imbalance and obesity.* Cell, 2008. **135**(1): p. 61-73.

131. Purkayastha, S., G. Zhang, and D. Cai, *Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic IKK-beta and NF-kappaB.* Nat Med, 2011. **17**(7): p. 883-7.

132. Thaler, J.P., et al., *Obesity is associated with hypothalamic injury in rodents and humans.* J Clin Invest, 2012. **122**(1): p. 153-62.

133. Mayer, C.M. and D.D. Belsham, *Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation.* Endocrinology, 2010. **151**(2): p. 576-85.

134. Moraes, J.C., et al., *High-fat diet induces apoptosis of hypothalamic neurons.* PLoS One, 2009. **4**(4): p. e5045.

135. McNay, D.E., et al., *Remodeling of the arcuate nucleus energy-balance circuit is inhibited in obese mice.* J Clin Invest, 2012. **122**(1): p. 142-52.

136. Jin, S., et al., *Hypothalamic TLR2 triggers sickness behavior via a microglia-neuronal axis.* Sci Rep, 2016. **6**: p. 29424.

137. Hopkins, S.J. and N.J. Rothwell, *Cytokines and the nervous system. I: Expression and recognition.* Trends Neurosci, 1995. **18**(2): p. 83-8.

138. Rothwell, N.J. and S.J. Hopkins, *Cytokines and the nervous system II: Actions and mechanisms of action.* Trends Neurosci, 1995. **18**(3): p. 130-6.

139. Licinio, J. and M.L. Wong, *Pathways and mechanisms for cytokine signaling of the central nervous system.* J Clin Invest, 1997. **100**(12): p. 2941-7.

140. Sternberg, E.M., *Neural-immune interactions in health and disease.* J Clin Invest, 1997. **100**(11): p. 2641-7.

141. Haslett, P.A., *Anticytokine approaches to the treatment of anorexia and cachexia.* Semin Oncol, 1998. **25**(2 Suppl 6): p. 53-7.

142. Mantovani, G., et al., *Cytokine activity in cancer-related anorexia/cachexia: role of megestrol acetate and medroxyprogesterone acetate.* Semin Oncol, 1998. **25**(2 Suppl 6): p. 45-52.

143. Gelin, J., et al., *Role of endogenous tumor necrosis factor alpha and interleukin 1 for experimental tumor growth and the development of cancer cachexia.* Cancer Res, 1991. **51**(1): p. 415-21.

144. Moldawer, L.L., M.A. Rogy, and S.F. Lowry, *The role of cytokines in cancer cachexia.* JPEN J Parenter Enteral Nutr, 1992. **16**(6 Suppl): p. 43S-49S.

145. Matthys, P. and A. Billiau, *Cytokines and cachexia.* Nutrition, 1997. **13**(9): p. 763-70.

146. Tisdale, M.J., *Biology of cachexia.* J Natl Cancer Inst, 1997. **89**(23): p. 1763-73.

147. Sherry, B.A., et al., *Anticachectin/tumor necrosis factor-alpha antibodies attenuate development of cachexia in tumor models.* FASEB J, 1989. **3**(8): p. 1956-62.

148. Noguchi, Y., et al., *Are cytokines possible mediators of cancer cachexia?* Surg Today, 1996. **26**(7): p. 467-75.

149. Ropelle, E.R., et al., *A central role for neuronal adenosine 5'-monophosphate-activated protein kinase in cancer-induced anorexia.* Endocrinology, 2007. **148**(11): p. 5220-9.

150. Lira, F.S., et al., *Hypothalamic inflammation is reversed by endurance training in anorectic-cachectic rats.* Nutr Metab (Lond), 2011. **8**(1): p. 60.

151. Deboer, M.D., et al., *Ghrelin treatment of chronic kidney disease: improvements in lean body mass and cytokine profile.* Endocrinology, 2008. **149**(2): p. 827-35.

152. DeBoer, M.D., et al., *Ghrelin treatment causes increased food intake and retention of lean body mass in a rat model of cancer cachexia.* Endocrinology, 2007. **148**(6): p. 3004-12.

153. Smeitink, J., L. van den Heuvel, and S. DiMauro, *The genetics and pathology of oxidative phosphorylation.* Nat Rev Genet, 2001. **2**(5): p. 342-52.

154. Okado-Matsumoto, A. and I. Fridovich, *Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria.* J Biol Chem, 2001. **276**(42): p. 38388-93.

155. Lebovitz, R.M., et al., *Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice.* Proc Natl Acad Sci U S A, 1996. **93**(18): p. 9782-7.

156. Li, Y., et al., *Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase.* Nat Genet, 1995. **11**(4): p. 376-81.

157. Zhang, D.X. and D.D. Gutterman, *Mitochondrial reactive oxygen species-mediated signaling in endothelial cells.* Am J Physiol Heart Circ Physiol, 2007. **292**(5): p. H2023-31.

158. Starkov, A.A., *The role of mitochondria in reactive oxygen species metabolism and signaling.* Ann N Y Acad Sci, 2008. **1147**: p. 37-52.

159. Gibson, G.E., et al., *Cause and consequence: mitochondrial dysfunction initiates and propagates neuronal dysfunction, neuronal death and behavioral abnormalities in age-associated neurodegenerative diseases.* Biochim Biophys Acta, 2010. **1802**(1): p. 122-34.

160. Schonfeld, P. and L. Wojtczak, *Fatty acids as modulators of the cellular production of reactive oxygen species.* Free Radic Biol Med, 2008. **45**(3): p. 231-41.

161. Ramalingam, M. and S.J. Kim, *Reactive oxygen/nitrogen species and their functional correlations in neurodegenerative diseases.* J Neural Transm (Vienna), 2012. **119**(8): p. 891-910.

162. Rodrigues, R., R.B. Petersen, and G. Perry, *Parallels between major depressive disorder and Alzheimer's disease: role of oxidative stress and genetic vulnerability.* Cell Mol Neurobiol, 2014. **34**(7): p. 925-49.

163. Domingueti, C.P., et al., *Diabetes mellitus: The linkage between oxidative stress, inflammation, hypercoagulability and vascular complications.* J Diabetes Complications, 2016. **30**(4): p. 738-45.

164. Bhattacharyya, S. and J. Saha, *Tumour, Oxidative Stress and Host T Cell Response: Cementing the Dominance.* Scand J Immunol, 2015. **82**(6): p. 477-88.

165. Li, J.M. and A.M. Shah, *Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(5): p. R1014-30.

166. Bashan, N., et al., *Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species.* Physiol Rev, 2009. **89**(1): p. 27-71.

167. Benani, A., et al., *Role for mitochondrial reactive oxygen species in brain lipid sensing: redox regulation of food intake.* Diabetes, 2007. **56**(1): p. 152-60.

168. Leloup, C., et al., *Mitochondrial reactive oxygen species are required for hypothalamic glucose sensing.* Diabetes, 2006. **55**(7): p. 2084-90.

169. Hsieh, H.L. and C.M. Yang, *Role of redox signaling in neuroinflammation and neurodegenerative diseases.* Biomed Res Int, 2013. **2013**: p. 484613.

170. Ott, M., et al., *Mitochondria, oxidative stress and cell death.* Apoptosis, 2007. **12**(5): p. 913-22.

171. Robinson, B.H., *Lactic acidemia and mitochondrial disease.* Mol Genet Metab, 2006. **89**(1-2): p. 3-13.

172. Distelmaier, F., et al., *Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease.* Brain, 2009. **132**(Pt 4): p. 833-42.

173. Drose, S. and U. Brandt, *Molecular mechanisms of superoxide production by the mitochondrial respiratory chain.* Adv Exp Med Biol, 2012. **748**: p. 145-69.

174. Perier, C., et al., *Complex I deficiency primes Bax-dependent neuronal apoptosis through mitochondrial oxidative damage.* Proc Natl Acad Sci U S A, 2005. **102**(52): p. 19126-31.

175. Boffoli, D., et al., *Decline with age of the respiratory chain activity in human skeletal muscle.* Biochim Biophys Acta, 1994. **1226**(1): p. 73-82.

176. Brieger, K., et al., *Reactive oxygen species: from health to disease.* Swiss Med Wkly, 2012. **142**: p. w13659.

177. Mattson, M.P. and D. Liu, *Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders.* Neuromolecular Med, 2002. **2**(2): p. 215-31.

178. Brandt, U., *Energy converting NADH:quinone oxidoreductase (complex I).* Annu Rev Biochem, 2006. **75**: p. 69-92.

179. Hirst, J., *Mitochondrial complex I.* Annu Rev Biochem, 2013. **82**: p. 551-75.

180. Ugalde, C., et al., *Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies.* Hum Mol Genet, 2004. **13**(20): p. 2461-72.

181. Balsa, E., et al., *NDUFA4 is a subunit of complex IV of the mammalian electron transport chain.* Cell Metab, 2012. **16**(3): p. 378-86.

182. Hirst, J., et al., *The nuclear encoded subunits of complex I from bovine heart mitochondria.* Biochim Biophys Acta, 2003. **1604**(3): p. 135-50.

183. Carroll, J., et al., *Bovine complex I is a complex of 45 different subunits.* J Biol Chem, 2006. **281**(43): p. 32724-7.

184. Wiedemann, N., A.E. Frazier, and N. Pfanner, *The protein import machinery of mitochondria.* J Biol Chem, 2004. **279**(15): p. 14473-6.

185. Nouws, J., et al., *Assembly factors as a new class of disease genes for mitochondrial complex I deficiency: cause, pathology and treatment options.* Brain, 2012. **135**(Pt 1): p. 12-22.

186. Kirby, D.M., et al., *NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency.* J Clin Invest, 2004. **114**(6): p. 837-45.

187. McFarland, R., et al., *De novo mutations in the mitochondrial ND3 gene as a cause of infantile mitochondrial encephalopathy and complex I deficiency.* Ann Neurol, 2004. **55**(1): p. 58-64.

188. Rodenburg, R.J., *Mitochondrial complex I-linked disease.* Biochim Biophys Acta, 2016. **1857**(7): p. 938-45.

189. Koopman, W.J., et al., *Mitochondrial disorders in children: toward development of small-molecule treatment strategies.* EMBO Mol Med, 2016. **8**(4): p. 311-27.

190. Fassone, E. and S. Rahman, *Complex I deficiency: clinical features, biochemistry and molecular genetics.* J Med Genet, 2012. **49**(9): p. 578-90.

191. Darin, N., et al., *The incidence of mitochondrial encephalomyopathies in childhood: clinical features and morphological, biochemical, and DNA abnormalities.* Ann Neurol, 2001. **49**(3): p. 377-83.

192. Leigh, D., *Subacute necrotizing encephalomyelopathy in an infant.* J Neurol Neurosurg Psychiatry, 1951. **14**(3): p. 216-21.

193. Rahman, S., et al., *Leigh syndrome: clinical features and biochemical and DNA abnormalities.* Ann Neurol, 1996. **39**(3): p. 343-51.

194. Luft, R. and B.R. Landau, *Mitochondrial medicine.* J Intern Med, 1995. **238**(5): p. 405-21.

195. Giulivi, C., et al., *Mitochondrial dysfunction in autism.* JAMA, 2010. **304**(21): p. 2389-96.

196. Cikankova, T., et al., *Mitochondrial dysfunctions in bipolar disorder: effect of the disease and pharmacotherapy.* CNS Neurol Disord Drug Targets, 2016.

197. Victor, V.M., et al., *Altered mitochondrial function and oxidative stress in leukocytes of anorexia nervosa patients.* PLoS One, 2014. **9**(9): p. e106463.

198. Kirby, D.M., et al., *Respiratory chain complex I deficiency: an underdiagnosed energy generation disorder.* Neurology, 1999. **52**(6): p. 1255-64.

199. Calvo, S.E., et al., *High-throughput, pooled sequencing identifies mutations in NUBPL and FOXRED1 in human complex I deficiency.* Nat Genet, 2010. **42**(10): p. 851-8.

200. Thorburn, D.R., et al., *Biochemical and molecular diagnosis of mitochondrial respiratory chain disorders.* Biochim Biophys Acta, 2004. **1659**(2-3): p. 121-8.

201. Vogel, R.O., et al., *Human mitochondrial complex I assembly is mediated by NDUFAF1.* FEBS J, 2005. **272**(20): p. 5317-26.

202. Ogilvie, I., N.G. Kennaway, and E.A. Shoubridge, *A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy.* J Clin Invest, 2005. **115**(10): p. 2784-92.

203. Vogel, R.O., et al., *Identification of mitochondrial complex I assembly intermediates by tracing tagged NDUFS3 demonstrates the entry point of mitochondrial subunits.* J Biol Chem, 2007. **282**(10): p. 7582-90.

204. Vogel, R.O., et al., *Cytosolic signaling protein Ecsit also localizes to mitochondria where it interacts with chaperone NDUFAF1 and functions in complex I assembly.* Genes Dev, 2007. **21**(5): p. 615-24.

205. Vogel, R.O., et al., *Investigation of the complex I assembly chaperones B17.2L and NDUFAF1 in a cohort of CI deficient patients.* Mol Genet Metab, 2007. **91**(2): p. 176-82.

206. Saada, A., et al., *C6ORF66 is an assembly factor of mitochondrial complex I.* Am J Hum Genet, 2008. **82**(1): p. 32-8.

207. Andrews, B., et al., *Assembly factors for the membrane arm of human complex I.* Proc Natl Acad Sci U S A, 2013. **110**(47): p. 18934-9.

208. Formosa, L.E., et al., *Characterization of mitochondrial FOXRED1 in the assembly of respiratory chain complex I.* Hum Mol Genet, 2015. **24**(10): p. 2952-65.

209. Heide, H., et al., *Complexome profiling identifies TMEM126B as a component of the mitochondrial complex I assembly complex.* Cell Metab, 2012. **16**(4): p. 538-49.

210. McKenzie, M. and M.T. Ryan, *Assembly factors of human mitochondrial complex I and their defects in disease.* IUBMB Life, 2010. **62**(7): p. 497-502.

211. Janssen, R., et al., *CIA30 complex I assembly factor: a candidate for human complex I deficiency?* Hum Genet, 2002. **110**(3): p. 264-70.

212. Guerrero-Castillo, S., et al., *The Assembly Pathway of Mitochondrial Respiratory Chain Complex I.* Cell Metab, 2016.

213. Mick, D.U., et al., *MITRAC links mitochondrial protein translocation to respiratory-chain assembly and translational regulation.* Cell, 2012. **151**(7): p. 1528-41.

214. Dunning, C.J., et al., *Human CIA30 is involved in the early assembly of mitochondrial complex I and mutations in its gene cause disease.* EMBO J, 2007. **26**(13): p. 3227-37.

215. Fassone, E., et al., *Mutations in the mitochondrial complex I assembly factor NDUFAF1 cause fatal infantile hypertrophic cardiomyopathy.* J Med Genet, 2011. **48**(10): p. 691-7.

216. Wu, L., et al., *Leukodystrophy associated with mitochondrial complex I deficiency due to a novel mutation in the NDUFAF1 gene.* Mitochondrial DNA A DNA Mapp Seq Anal, 2016. **27**(2): p. 1034-7.

217. Barghuti, F., et al., *The unique neuroradiology of complex I deficiency due to NDUFA12L defect.* Mol Genet Metab, 2008. **94**(1): p. 78-82.

218. Hoefs, S.J., et al., *Baculovirus complementation restores a novel NDUFAF2 mutation causing complex I deficiency.* Hum Mutat, 2009. **30**(7): p. E728-36.

219. Janssen, R.J., et al., *Contiguous gene deletion of ELOVL7, ERCC8 and NDUFAF2 in a patient with a fatal multisystem disorder.* Hum Mol Genet, 2009. **18**(18): p. 3365-74.

220. Herzer, M., et al., *Leigh disease with brainstem involvement in complex I deficiency due to assembly factor NDUFAF2 defect.* Neuropediatrics, 2010. **41**(1): p. 30-4.

221. Ghaloul-Gonzalez, L., et al., *Mitochondrial respiratory chain disorders in the Old Order Amish population.* Mol Genet Metab, 2016. **118**(4): p. 296-303.

222. Saada, A., et al., *Mutations in NDUFAF3 (C3ORF60), encoding an NDUFAF4 (C6ORF66)-interacting complex I assembly protein, cause fatal neonatal mitochondrial disease.* Am J Hum Genet, 2009. **84**(6): p. 718-27.

223. Sugiana, C., et al., *Mutation of C20orf7 disrupts complex I assembly and causes lethal neonatal mitochondrial disease.* Am J Hum Genet, 2008. **83**(4): p. 468-78.

224. Gerards, M., et al., *Defective complex I assembly due to C20orf7 mutations as a new cause of Leigh syndrome.* J Med Genet, 2010. **47**(8): p. 507-12.

225. Saada, A., et al., *Combined OXPHOS complex I and IV defect, due to mutated complex I assembly factor C20ORF7.* J Inherit Metab Dis, 2012. **35**(1): p. 125-31.

226. Pagliarini, D.J., et al., *A mitochondrial protein compendium elucidates complex I disease biology.* Cell, 2008. **134**(1): p. 112-23.

227. Carilla-Latorre, S., et al., *MidA is a putative methyltransferase that is required for mitochondrial complex I function.* J Cell Sci, 2010. **123**(Pt 10): p. 1674-83.

228. Nouws, J., et al., *Acyl-CoA dehydrogenase 9 is required for the biogenesis of oxidative phosphorylation complex I.* Cell Metab, 2010. **12**(3): p. 283-94.

229. Gerards, M., et al., *Riboflavin-responsive oxidative phosphorylation complex I deficiency caused by defective ACAD9: new function for an old gene.* Brain, 2011. **134**(Pt 1): p. 210-9.

230. Haack, T.B., et al., *Exome sequencing identifies ACAD9 mutations as a cause of complex I deficiency.* Nat Genet, 2010. **42**(12): p. 1131-4.

231. Scholte, H.R., et al., *Riboflavin-responsive complex I deficiency.* Biochim Biophys Acta, 1995. **1271**(1): p. 75-83.

232. Collet, M., et al., *High incidence and variable clinical outcome of cardiac hypertrophy due to ACAD9 mutations in childhood.* Eur J Hum Genet, 2016. **24**(8): p. 1112-6.

233. Dewulf, J.P., et al., *Evidence of a wide spectrum of cardiac involvement due to ACAD9 mutations: Report on nine patients.* Mol Genet Metab, 2016. **118**(3): p. 185-9.

234. Pronicka, E., et al., *New perspective in diagnostics of mitochondrial disorders: two years' experience with whole-exome sequencing at a national paediatric centre.* J Transl Med, 2016. **14**(1): p. 174.

235. Alston, C.L., et al., *Biallelic Mutations in TMEM126B Cause Severe Complex I Deficiency with a Variable Clinical Phenotype.* Am J Hum Genet, 2016. **99**(1): p. 217-27.

236. Sheftel, A.D., et al., *Human ind1, an iron-sulfur cluster assembly factor for respiratory complex I.* Mol Cell Biol, 2009. **29**(22): p. 6059-73.

237. Calvo, S.E., et al., *Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing.* Sci Transl Med, 2012. **4**(118): p. 118ra10.

238. Tucker, E.J., et al., *Next-generation sequencing in molecular diagnosis: NUBPL mutations highlight the challenges of variant detection and interpretation.* Hum Mutat, 2012. **33**(2): p. 411-8.

239. Kevelam, S.H., et al., *NUBPL mutations in patients with complex I deficiency and a distinct MRI pattern.* Neurology, 2013. **80**(17): p. 1577-83.

240. Fassone, E., et al., *FOXRED1, encoding an FAD-dependent oxidoreductase complex-I-specific molecular chaperone, is mutated in infantile-onset mitochondrial encephalopathy.* Hum Mol Genet, 2010. **19**(24): p. 4837-47.

241. Zurita Rendon, O., et al., *The arginine methyltransferase NDUFAF7 is essential for complex I assembly and early vertebrate embryogenesis.* Hum Mol Genet, 2014. **23**(19): p. 5159-70.

242. Jonckheere, A.I., et al., *Restoration of complex V deficiency caused by a novel deletion in the human TMEM70 gene normalizes mitochondrial morphology.* Mitochondrion, 2011. **11**(6): p. 954-63.

243. Belanger, M., I. Allaman, and P.J. Magistretti, *Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation.* Cell Metab, 2011. **14**(6): p. 724-38.

244. Beal, M.F., *Coenzyme Q10 as a possible treatment for neurodegenerative diseases.* Free Radic Res, 2002. **36**(4): p. 455-60.

245. Lin, M.T. and M.F. Beal, *Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases.* Nature, 2006. **443**(7113): p. 787-95.

246. Tillement, L., L. Lecanu, and V. Papadopoulos, *Alzheimer's disease: effects of beta-amyloid on mitochondria.* Mitochondrion, 2011. **11**(1): p. 13-21.

247. Butterfield, D.A., E.M. Palmieri, and A. Castegna, *Clinical implications from proteomic studies in neurodegenerative diseases: lessons from mitochondrial proteins.* Expert Rev Proteomics, 2016. **13**(3): p. 259-74.

248. Schapira, A.H., et al., *Mitochondrial complex I deficiency in Parkinson's disease.* J Neurochem, 1990. **54**(3): p. 823-7.

249. Parker, W.D., Jr., S.J. Boyson, and J.K. Parks, *Abnormalities of the electron transport chain in idiopathic Parkinson's disease.* Ann Neurol, 1989. **26**(6): p. 719-23.

250. Keeney, P.M., et al., *Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled.* J Neurosci, 2006. **26**(19): p. 5256-64.

251. Gatt, A.P., et al., *Dementia in Parkinson's disease is associated with enhanced mitochondrial complex I deficiency.* Mov Disord, 2016. **31**(3): p. 352-9.

252. Haelterman, N.A., et al., *A mitocentric view of Parkinson's disease.* Annu Rev Neurosci, 2014. **37**: p. 137-59.

253. Maechler, P., *Novel regulation of insulin secretion: the role of mitochondria.* Curr Opin Investig Drugs, 2003. **4**(10): p. 1166-72.

254. Rutter, G.A., *Nutrient-secretion coupling in the pancreatic islet beta-cell: recent advances.* Mol Aspects Med, 2001. **22**(6): p. 247-84.

255. Namkoong, C., et al., *Enhanced hypothalamic AMP-activated protein kinase activity contributes to hyperphagia in diabetic rats.* Diabetes, 2005. **54**(1): p. 63-8.

256. Abdulreda, M.H. and P.O. Berggren, *Islet inflammation in plain sight.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 105-16.

257. Arruda, A.P., et al., *Low-grade hypothalamic inflammation leads to defective thermogenesis, insulin resistance, and impaired insulin secretion.* Endocrinology, 2011. **152**(4): p. 1314-26.

258. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease.* Nat Rev Immunol, 2011. **11**(2): p. 98-107.

259. Donath, M.Y., *Targeting inflammation in the treatment of type 2 diabetes.* Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 193-6.

260. Thaler, J.P., et al., *Hypothalamic inflammation and energy homeostasis: resolving the paradox.* Front Neuroendocrinol, 2010. **31**(1): p. 79-84.

261. Velloso, L.A., E.P. Araujo, and C.T. de Souza, *Diet-induced inflammation of the hypothalamus in obesity.* Neuroimmunomodulation, 2008. **15**(3): p. 189-93.

262. Johansen, J.E., et al., *Hypothalamic CART and serum leptin levels are reduced in the anorectic (anx/anx) mouse.* Brain Res Mol Brain Res, 2000. **84**(1-2): p. 97-105.

263. Broberger, C., et al., *Changes in neuropeptide Y receptors and pro-opiomelanocortin in the anorexia (anx/anx) mouse hypothalamus.* J Neurosci, 1999. **19**(16): p. 7130-9.

264. Son, J.H., et al., *Drastic and selective hyperinnervation of central serotonergic neurons in a lethal neurodevelopmental mouse mutant, Anorexia (anx).* Brain Res Mol Brain Res, 1994. **25**(1-2): p. 129-34.

265. Broberger, C., *Hypothalamic cocaine- and amphetamine-regulated transcript (CART) neurons: histochemical relationship to thyrotropin-releasing hormone, melanin-concentrating hormone, orexin/hypocretin and neuropeptide Y.* Brain Res, 1999. **848**(1-2): p. 101-13.

266. Broberger, C., et al., *Hypothalamic neurohistochemistry of the murine anorexia (anx/anx) mutation: altered processing of neuropeptide Y in the arcuate nucleus.* J Comp Neurol, 1997. **387**(1): p. 124-35.

267. Broberger, C., et al., *Subtypes Y1 and Y2 of the neuropeptide Y receptor are respectively expressed in pro-opiomelanocortin- and neuropeptide-Y-containing neurons of the rat hypothalamic arcuate nucleus.* Neuroendocrinology, 1997. **66**(6): p. 393-408.

268. Fetissov, S.O., et al., *Alterations of arcuate nucleus neuropeptidergic development in contactin-deficient mice: comparison with anorexia and food-deprived mice.* Eur J Neurosci, 2005. **22**(12): p. 3217-28.

269. Johansen, J.E., et al., *Altered dopaminergic transmission in the anorexic anx/anx mouse striatum.* Neuroreport, 2001. **12**(12): p. 2737-41.

270. Jahng, J.W., et al., *Neuropeptide Y mRNA and serotonin innervation in the arcuate nucleus of anorexia mutant mice.* Brain Res, 1998. **790**(1-2): p. 67-73.

271. Jahng, J.W., et al., *Differential expression of monoamine oxidase A, serotonin transporter, tyrosine hydroxylase and norepinephrine transporter mRNA by anorexia mutation and food deprivation.* Brain Res Dev Brain Res, 1998. **107**(2): p. 241-6.

272. Dryden, S., et al., *The serotonergic agent fluoxetine reduces neuropeptide Y levels and neuropeptide Y secretion in the hypothalamus of lean and obese rats.* Neuroscience, 1996. **72**(2): p. 557-66.

273. Lachuer, J., et al., *Gene expression profiling reveals an inflammatory process in the anx/anx mutant mice.* Brain Res Mol Brain Res, 2005. **139**(2): p. 372-6.

274. Mercader, J.M., et al., *Hypothalamus transcriptome profile suggests an anorexia-cachexia syndrome in the anx/anx mouse model.* Physiol Genomics, 2008. **35**(3): p. 341-50.

275. Kim, M.J., et al., *Increases in cell proliferation and apoptosis in dentate gyrus of anorexia (anx/anx) mice.* Neurosci Lett, 2001. **302**(2-3): p. 109-12.

276. Chun, H.S., et al., *Identification of genes showing differential expression in anorexia mutant mouse.* Neuroreport, 2003. **14**(7): p. 1055-9.

277. Mercader, J.M., et al., *Association of NTRK3 and its interaction with NGF suggest an altered cross-regulation of the neurotrophin signaling pathway in eating disorders.* Hum Mol Genet, 2008. **17**(9): p. 1234-44.

278. Kim, D.Y., et al., *The tyrosine kinase receptor Tyro3 enhances lifespan and neuropeptide Y (Npy) neuron survival in the mouse anorexia (anx) mutation.* Dis Model Mech, 2017.

279. Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson, *Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation.* J Cell Biol, 1992. **119**(3): p. 493-501.

280. Andrews, Z.B., et al., *Uncoupling protein-2 is critical for nigral dopamine cell survival in a mouse model of Parkinson's disease.* J Neurosci, 2005. **25**(1): p. 184-91.

281. Lapidus, R.G. and P.M. Sokolove, *Spermine inhibition of the permeability transition of isolated rat liver mitochondria: an investigation of mechanism.* Arch Biochem Biophys, 1993. **306**(1): p. 246-53.

282. Lernmark, A., *The preparation of, and studies on, free cell suspensions from mouse pancreatic islets.* Diabetologia, 1974. **10**(5): p. 431-8.

283. Alp, P.R., E.A. Newsholme, and V.A. Zammit, *Activities of citrate synthase and NAD+-linked and NADP+-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates.* Biochem J, 1976. **154**(3): p. 689-700.

284. Wong, K.P., et al., *Effects of administration route, dietary condition, and blood glucose level on kinetics and uptake of 18F-FDG in mice.* J Nucl Med, 2011. **52**(5): p. 800-7.

285. Imai, Y., et al., *A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage.* Biochem Biophys Res Commun, 1996. **224**(3): p. 855-62.

286. Perry, V.H., D.A. Hume, and S. Gordon, *Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain.* Neuroscience, 1985. **15**(2): p. 313-26.

287. Streit, W.J., M.B. Graeber, and G.W. Kreutzberg, *Functional plasticity of microglia: a review.* Glia, 1988. **1**(5): p. 301-7.

288. Elward, K. and P. Gasque, *"Eat me" and "don't eat me" signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system.* Mol Immunol, 2003. **40**(2-4): p. 85-94.

289. Ribak, C.E., et al., *Microglia-associated granule cell death in the normal adult dentate gyrus.* Brain Struct Funct, 2009. **214**(1): p. 25-35.

290. Neumann, H., et al., *Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha.* J Exp Med, 1997. **185**(2): p. 305-16.

291. Corriveau, R.A., G.S. Huh, and C.J. Shatz, *Regulation of class I MHC gene expression in the developing and mature CNS by neural activity.* Neuron, 1998. **21**(3): p. 505-20.

292. Oliveira, A.L., et al., *A role for MHC class I molecules in synaptic plasticity and regeneration of neurons after axotomy.* Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17843-8.

293. Frenois, F., et al., *Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior.* Psychoneuroendocrinology, 2007. **32**(5): p. 516-31.

294. Nikolaev, A., et al., *APP binds DR6 to trigger axon pruning and neuron death via distinct caspases.* Nature, 2009. **457**(7232): p. 981-9.

295. Graham, R.K., et al., *Levels of mutant huntingtin influence the phenotypic severity of Huntington disease in YAC128 mouse models.* Neurobiol Dis, 2006. **21**(2): p. 444-55.

296. Andrews, Z.B., et al., *UCP2 mediates ghrelin's action on NPY/AgRP neurons by lowering free radicals.* Nature, 2008. **454**(7206): p. 846-51.

297. Horvath, T.L., Z.B. Andrews, and S. Diano, *Fuel utilization by hypothalamic neurons: roles for ROS.* Trends Endocrinol Metab, 2009. **20**(2): p. 78-87.

298. Neumann, H., et al., *Induction of MHC class I genes in neurons.* Science, 1995. **269**(5223): p. 549-52.

299. Chandrasekaran, K., et al., *Evidence for physiological down-regulation of brain oxidative phosphorylation in Alzheimer's disease.* Exp Neurol, 1996. **142**(1): p. 80-8.

300. Tenney, J.R., et al., *Cerebral glucose hypometabolism is associated with mitochondrial dysfunction in patients with intractable epilepsy and cortical dysplasia.* Epilepsia, 2014. **55**(9): p. 1415-22.

301. Cunnane, S., et al., *Brain fuel metabolism, aging, and Alzheimer's disease.* Nutrition, 2011. **27**(1): p. 3-20.

302. Ehses, J.A., et al., *IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13998-4003.

303. Masters, S.L., et al., *Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes.* Nat Immunol, 2010. **11**(10): p. 897-904.

304. Pinter, E.J., G. Tolis, and H.G. Friesen, *L-dopa, growth hormone and adipokinesis in the lean and the obese.* Int J Clin Pharmacol Biopharm, 1975. **12**(1-2): p. 277-80.

305. Curatola, G., et al., *Chemical-physical properties of lipoproteins in anorexia nervosa.* Eur J Clin Invest, 2004. **34**(11): p. 747-51.

306. Jauregui-Garrido, B., et al., *Lipid profile and cardiovascular risk in anorexia nervosa; the effect of nutritional treatment.* Nutr Hosp, 2012. **27**(3): p. 908-13.

307. Yin, J., et al., *Toll-like receptor 2/4 links to free fatty acid-induced inflammation and beta-cell dysfunction.* J Leukoc Biol, 2014. **95**(1): p. 47-52.

308. Eguchi, K., et al., *Saturated fatty acid and TLR signaling link beta cell dysfunction and islet inflammation.* Cell Metab, 2012. **15**(4): p. 518-33.

309. Gianotti, L., et al., *Effects of free fatty acids and acipimox, a lipolysis inhibitor, on the somatotroph responsiveness to GHRH in anorexia nervosa.* Clin Endocrinol (Oxf), 2000. **52**(6): p. 713-20.

310. Dunn-Meynell, A.A., N.E. Rawson, and B.E. Levin, *Distribution and phenotype of neurons containing the ATP-sensitive K+ channel in rat brain.* Brain Res, 1998. **814**(1-2): p. 41-54.

311. Ibrahim, N., et al., *Hypothalamic proopiomelanocortin neurons are glucose responsive and express K(ATP) channels.* Endocrinology, 2003. **144**(4): p. 1331-40.

312. Thomzig, A., et al., *Pore-forming subunits of K-ATP channels, Kir6.1 and Kir6.2, display prominent differences in regional and cellular distribution in the rat brain.* J Comp Neurol, 2005. **484**(3): p. 313-30.

313. Ashcroft, F.M., *Adenosine 5'-triphosphate-sensitive potassium channels.* Annu Rev Neurosci, 1988. **11**: p. 97-118.

314. Liss, B., R. Bruns, and J. Roeper, *Alternative sulfonylurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons.* EMBO J, 1999. **18**(4): p. 833-46.

315. van den Top, M., et al., *Pharmacological and molecular characterization of ATP-sensitive K(+) conductances in CART and NPY/AgRP expressing neurons of the hypothalamic arcuate nucleus.* Neuroscience, 2007. **144**(3): p. 815-24.

316. Ashcroft, S.J. and F.M. Ashcroft, *Properties and functions of ATP-sensitive K-channels.* Cell Signal, 1990. **2**(3): p. 197-214.

317. Blondeau, N., et al., *K(ATP) channel openers, adenosine agonists and epileptic preconditioning are stress signals inducing hippocampal neuroprotection.* Neuroscience, 2000. **100**(3): p. 465-74.

318. Chergui, K., et al., *Increased expression of NGFI-A mRNA in the rat striatum following burst stimulation of the medial forebrain bundle.* Eur J Neurosci, 1997. **9**(11): p. 2370-82.

319. Liss, B. and J. Roeper, *Molecular physiology of neuronal K-ATP channels (review).* Mol Membr Biol, 2001. **18**(2): p. 117-27.